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WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, 9/88, A01H 5/00 | A1

(11) International Publication Number: WO 92/08794

(43) International Publication Date: 29 May 1992 (29.05.92)

(21) International Application Number: PCT/GB91/01972

(22) International Filing Date: 8 November 1991 (08.11.91)

(30) Priority data:
9024728.9 14 November 1990 (14.11.90) GB

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: HERBICIDE RESISTANT PLANTS

(57) Abstract

A DNA coding for a mutant acetolactate synthase enzyme which confers, on a plant into which it is introduced by transformation, resistance to herbicides which normally inhibit wild-type acetolactate synthase such as herbicides of the imidazolinone and sulphonylurea families, has the sequence shown in Figure 24 or Figure 25 of the drawings.

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HERBICIDE RESISTANT PLANTS

This invention relates to herbicide resistant maize plants and mutant gene sequences providing said resistance. The invention further relates to plants containing said gene sequences and the seed and progeny of the plants.

The purpose in providing crop plants which resist the action of a herbicide is to facilitate the destruction of weeds growing between the plants by the overall application of a herbicidally effective concentration of a herbicide which would destroy the crop plant in its normal, that is herbicide sensitive, state. Such resistant plants are also useful for use in a locus of any short term carry-over of herbicide from a previous crop.

Methods are known by which populations of plants may be obtained which contain a great number of random mutations. Such methods include tissue culture techniques where spontaneous somaclonal variation occurs in the presence or absence of a mutagen. By applying to the cultures some form of selection pressure it is possible to recover cells which resist that pressure. Depending on the plant species it is sometimes possible to regenerate whole plants from the resistant cells. Such tissue culture selection methods have been used in the past to select for resistance to herbicides.

Neuffer and Coe [Maydica XXIII (1978) 21-28; page 21] have described a procedure for corn

(maize) pollen mutagenesis using mutagens suspended in light paraffin oil, followed by pollination of a recipient plant with the mutagenized pollen. As reported, there is no indication that any attempt was ever made to locate and isolate commercially important mutants and, although it is said that the resulting plants were examined for mutants, no details are given as to the properties of any mutants which may have been found, probably by visual inspection.

In Plant Breeding Reviews 5, pages 39 to 180, Bird and Neuffer review the uses of mutagenic processes to produce variation in maize. Pollen mutagenesis is discussed at pages 150 and 151. Pollen mutageneseis gives a relatively high frequency of variation in the M_1 generation compared with other available procedures. However, no suggestion is made in respect of the use of pollen mutagenesis for generation of mutants which display resistance to herbicide action. Section III (page 149) describes some of the difficulties of the use of mutagenesis as a source of genetic variation. One important aspect is the method by which the population of putative mutants is screened for useful phenotypes.

Our co-pending International Patent Application Number PCT/GB 90/00753 describes and claims maize plants which are resistant to the effects of imidazolinone and/or sulphonyl urea herbicides. These plants were produced by a pollen mutagenesis method and are surprisingly free from deleterious mutations which would be expected from a procedure such as chemical mutagenesis which is generally considered to act randomly.

Although mutagenesis or somaclonal variation may provide plants of a particular species which are resistant to a selected herbicide, the resistant trait cannot be transferred to other plant species. Nor, indeed, can the trait be transferred to other plant varieties within the same species without an extended and difficult breeding programme.

European Patent Application Number 257,993 discloses a general scheme of mutations to the gene encoding acetolactate synthase which are said to confer resistance to the sulphonylurea class of herbicides.

An object of the present invention is to provide genetic material for using in imparting herbicide resistance to plants.

According to the present invention there is provided a DNA encoding a mutant acetolactate synthase and having the nucleotide sequence shown in Figure 24 or 25 of the drawings herewith, and variations thereof permitted by the degeneracy of the genetic code.

A plant DNA having a nucleotide sequence having homology with that shown in Figure 1 and which contains a mutation at a position equivalent to number 171 in Figure 24 and/or number 1888 in Figure 25.

A plant DNA comprising the Apal to Stul or the Xhol to Stul restriction fragment shown in Figure 24 or the Xhol to NspH1 restriction fragment shown in Figure 25, and variations in said DNAs as permitted by the degeneracy of the genetic code.

Plants, particularly maize plants, which contain said DNAs stably integrated within their

genomes.

The invention also provides seeds and progeny of the said plant which have been produced by crossing of the plants of this invention with other maize plant lines.

The mutant DNAs of this invention may be isolated from seeds deposited on 8th May 1989 with the National Collection of Industrial & Marine Bacteria, Aberdeen, United Kingdom, under the Accession Numbers NCIMB 40137 and 40136. Each of these deposits is a genetic mixture of maize seeds, segregating mutant and non-mutant seeds. The mutants are heterozygous for the gene conferring herbicide resistance. Mutant plants may be derived from these seeds by growing under the conditions described below under the heading "Screening" in the presence of the herbicide imazethapyr.

The plants of this invention are known to be resistant to certain members of the imidazolinone family of herbicides, for example, imazethapyr [5-ethyl-2-(5-iso- propyl-5-methyl-4-oxo-2-imidazolin-2-yl) nicotinic acid: (Trade Mark PURSUIT, American Cyanamid)]. Cross resistance to herbicides of the sulphonylurea family, chlorsulfuron, for example, might have been expected on the basis of reports in the literature and the fact that acetolactate synthase is the site of action of both the imidazolinones and the sulphonylureas. However, we have found, quite unexpectedly that the resistance conferred by the genes of this invention is specific to the imidazolinones in general and PURSUIT particularly. Any tolerance which the plants may have of the sulphonylureas is insignificant.

A detailed exposition of the molecular basis

of the resistance to the sulphonylurea herbicides is given in European Patent Application 257,993 (E.I. Du Pont de Nemours and Company). Isolation of imidazolinone-resistant maize from tissue culture is reported in United States Patent Number 4,761,373 (Molecular Genetics Inc.). Various plant species and several herbicides are reported in the literature as having been used in tissue culture processes to isolate resistant mutants.

In comparison, source of the genes which are the principal subject of this invention have been isolated from maize plant to which the resistance mutations have been introduced by mutagenesis of pollen by a chemical mutagen. Selection was effected directly on the seeds formed after fertilisation with the mutagenised pollen either by treatment of the seed pre-emergence or at the seedling stage, or both.

Certain advantages accrue from the use of pollen mutagenesis as a method of creating mutants rather than the more usual method of relying on somaclonal variation to produce the variation. In order that somaclonal variation may occur it is required that a culture of plant tissue be established. This requirement restricts the choice of genotype which may be used as it is not always possible to regenerate whole plants from the cultured tissue. On the other hand, mutagenised pollen may be applied to any recipient maize genotype, including commercially important and well-established elite breeding lines. Also the rate of occurrence of undesirable mutations which somaclonal variation is known to produce is unexpectedly reduced.

Full details of the resistance and the manner in which it is created are given in our International Patent Application No PCT/GB90/00753 relevant portions of which are quoted below. The present invention is based largely on DNA sequence data of the genes present in our mutants numbered 1 and 2 as described in the said International Application.

International Patent Application No PCT/GB90/00753

Selection is carried out at at the M_1 generation, with the result that only dominant mutations are selected. Also, being carried out on whole plants or on the seed pre-emergence, or both, allows the herbicide concentration to mimic the field conditions more closely than is possible with the application of the selection pressure of the herbicide to a tissue culture. In the tissue culture selection method, whole plants have to be regenerated from the tissue and grown to maturity before any indication of the performance of the progeny under field application rates of the herbicide can be obtained. In our method, selection is made directly on the plants under concentrations of herbicide which are comparable to those which are recommended for normal weed-killing activity in the field. Selection on the M_2 generation, as with the tissue culture method, selects recessive mutations as well as dominant traits. Dominance of a desirable trait is generally viewed as more useful and easier to handle in a breeding programme especially of hybrid crops.

We believe that the mutants selected under herbicide pressure vary according to the particular

member of the herbicide family which is used. All of our mutants were selected under pressure of imazethapyr. Had a different imidazolinone herbicide been used, a different spectrum of mutants would have been selected.

We have found, quite surprisingly, that the mutants which we have isolated by the method of the invention are free of deleterious mutations. This was entirely unexpected and the reason for this advantage is not entirely clear. We believe, but do not wish to be bound by this explanation, that the degree of control which we are able to exercise over the selection step, using carefully controlled rates of application of the herbicide , for example, giving a good overall and uniform rate of exposure, may be responsible.

The invention will now be described by the following summary of the method by which the herbicide- resistant plants of the invention were derived.

The Figures which accompany this application are as follows:

Figure 1 is a flow-chart showing the derivation of several generations of progeny from plants generated by this invention;

Figure 2 shows the chemical structures of the herbicides used in this invention;

Figure 3 is a graph of ALS enzyme activity in the presence of imazethapyr. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 4 is a graph of ALS enzyme activity in the presence of imazapyr. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 5 is a graph of ALS enzyme activity in the presence of imazaquin. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 6 is a graph of ALS enzyme activity in the presence of chlorsulfuron. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 7 is a graph of ALS enzyme activity in the presence of chlorimuron. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 8 is a graph of ALS enzyme activity in the presence of thiacarburon. The enzyme is extracted from plants heterozygous for the resistance gene;

Figure 9 is an enzyme activity graph of the activity of ALS extracted from leaves of progeny of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of imazethapyr (PURSUIT);

Figure 10 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of imazaquin (SCEPTER);

Figure 11 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of imazapyr (ARSENAL);

Figure 12 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of chlorsulfuron (GLEAN);

Figure 13 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1

and 2 which are homozygous for the resistant allele and in the presence of chlorimuron (CLASSIC);

Figure 14 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of thiacarburon (HARMONY);

Figure 15 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2 which are homozygous for the resistant allele and in the presence of a triazolopyrimidine;

Figure 16 is an enzyme activity graph of the activity of ALS extracted from leaves of mutants 1 and 2-which are homozygous for the resistant allele and in the presence of a phenoxypyrimidine;

Figure 17 is a dose response curve for the herbicide imazethapyr (PURSUIT);

Figure 18 is a dose response curve for the herbicide imazaquin (SCEPTER);

Figure 19 is a dose response curve for the herbicide chlorimuron (CLASSIC);

Figure 20 is a dose response curve for the herbicide chlorsulfuron (GLEAN);

Figure 21 is a dose response curve for a triazolopyrimidine herbicide;

Figure 22 is a dose response curve for a phenoxypyrimidine herbicide; and,

Figure 23 is a dose response curve for the herbicide imazethapyr (PURSUIT) for mutants 1 and 2 in both heterozygous and homozygous forms.

1. <u>PRODUCTION OF M1 SEED</u>

A stock solution of ethyl methane sulphonate (EMS) was made up to contain one millilitre of EMS in 100 ml of light paraffin oil. The stock solution was stored under refrigeration.

Fresh pollen grains with anthers were harvested from a total of twenty tassels of field grown maize inbred line UE95. Pollen grains were separated from the anthers using a Glassine (Trade Mark) bag.

Around 3 milligrams of pollen were added to 45 millilitres of the EMS stock solution in a 60 ml capacity bottle. The pollen/EMS solution mixture was shaken vigorously for 30 seconds then shaken four or five times every three minutes over a period of 40 minutes, to prevent precipitation of the pollen grains. The treated pollen grains were brushed on to the silks of the detasseled inbred female parent (coded UE95).

The plants were grown to maturity and the M1 seeds harvested.

2. SCREENING

M1 seed was sown, 100 seeds per tray, in WCB growing medium (low organic matter, 45% loam, 55% grit) and sprayed to 'run-off' using a track sprayer, with a solution of imazethapyr (PURSUIT) at a concentration calculated to be the equivalent of 250 g/ha of active ingredient. The seeds were covered with 0.5 inch of WCB and grown in the glasshouse at 25°C.

The chosen application rate was such that germination was close to 100%, but subsequently all susceptible plants were severely affected. However, after about two weeks, the initial effect of the herbicide became apparent: thin striped leaves and reduced height of only about 20% of the height of the control plants. After three to four weeks almost all the sensitive plants were completely dead. Unsprayed UE95 plants were always

grown in parallel with each screen as a control as it was already known that normal and M1 seedlings of UE95 are almost indistinguishable when germinated and grown to maturity without spraying.

3. SELECTION AND GROWTH

A total of ten plants (representing 0.01% of the total) were initially selected from the screen as exhibiting tolerance of normally lethal dosages of the herbicide. Mutant No.4 was subsequently found to be of the sensitive phenotype and was withdrawn from the programme. Of the remaining nine, the majority were morphologically similar to the untreated control plants after selection but four were affected, being shorter and exhibiting other herbicidal effects. Samples of seeds of these nine plants back-crossed to the parent UE95 (Mutants 1 to 10, excluding No.4) are those which have been deposited with the National Collection of Industrial and Marine Bacteria (see Table 1 above).

4. RFLP STUDY

In order to confirm that the sibling plants were indeed of UE95 genotype in origin and not an intrinsically more resistant contaminating inbred or hybrid, RFLP (restriction fragment length polymorphism) fingerprinting analysis was performed on DNA extracted from leaf tissue from all ten plants.

Approximately 1 to 5 grams of leaf tissue was removed from each plant (ranging in age from four weeks to 'mature'), DNA extracted and RFLP analysis performed using diagnostic single copy probes.

RFLP analysis confirmed that the selected plants were of the UE95 genotype.

5. SEGREGATION STUDY

Resistant plants were used in reciprocal back-crosses with homozygous, herbicide sensitive UE95. The frequency of resistant progeny in the M_1BC_1 or M_1BC_2 generations was found by treatment with imazethapyr and counting the survivors. The results are shown in Table 2 below, along with the calculated value of X^2 (for a 1:1 ratio).

As can been seen from the figures quoted in Table 2, the ratio of resistant to sensitive plants in the progeny of the backcross is not significantly different from 1:1 for all of the mutants except numbers 3 and 7, indicating that resistance is controlled by a single dominant gene.

TABLE 2

Mutant No.	Number tested	Number sensitive	Number resistant	X^2 (for 1:1)
1	40	18	22	0.40
2	228	110	118	0.28
3☆	206	120	86	5.61
5	168	94	74	2.38
6	60	36	24	2.40
7☆	168	139	29	72.00
8	188	88	100	0.77
9	168	83	85	0.02
10	134	73	71	0.03

☆ The resistance of these mutants to imazethapyr is low and it was therefore difficult to make a meaningful assessment in this experiment but, experience in other tests indicates that segregation is in the region of 1:1.

6. GENERATION OF F1 SEED FROM RESISTANT PLANTS

All ten plants identified in the screen were used in reciprocal sib-crossing with UE95 plants to give

seed (designated M_1BC_1), that is, the resistant plants were used as both male and female donors.

7. RESCREENING OF THE PROGENY SEED

From each resulting cob, a small sample of seed was screened for imazethapyr (PURSUIT) resistance, employing the same conditions as are described above for the initial screen.

Resistant and sensitive phenotypes segregated in the progeny of each resistant plant.

8. PRODUCTION OF FURTHER GENERATIONS

Referring to Figure 1, the M_1BC_1 plants were self pollinated to give generation $M_1BC_1S_1$ which was again self-pollinated to give $M_1BC_1S_2$. From that generation it was possible to identify, by the fact that the resistant trait is non-segregating, lines which are homozygous for the trait. These homozygous lines derived from Mutant No.1 may be utilised for the production of F1 hybrids which possess resistance to the imidazolinone herbicides or for further breeding work.

9. ENZYME ASSAYS

Seeds of generation M_1BC_1 M_1BC_2 of the nine mutants were sprayed pre-emergence with the herbicide imazethapyr (PURSUIT) (Trade Mark) at a rate of 250 g/ha and grown in a growth chamber (16 hour day, 27°C; 8 hour night, 17°C). Plants were harvested after 11 days.

Four grams of leaf material were harvested from just above the first leaf axis and ground in a mortar and pestle in 20ml of a solution containing 40mM Tricine (Trade Mark), 10mM EDTA, 5mM pyruvate, 80 μM flavin adenine dinucleotide (FAD), 1mM dithiothreitol (DTT), pH 8, plus 0.8g Polyclar AT. The homogenate was pressed through four layers of muslin and

centrifuged at 30,000g for 20 minutes.

The supernatant was brought to 65% saturation with ammonium sulphate, left to precipitate for 30 minutes and then centrifuged at 30.000g for 20 minutes. The pellet was resuspended in 2.5ml of 40mM Tricine, 10mM EDTA, 5mM pyruvate, 80μM FAD, 25% (v/v) glycerol, 1mM DTT, pH 8, and desalted into 3.5ml of 40mM Tricine, 10mM EDTA, 25% (v/v) glycerol, 1mM DTT, pH 8.

One hundred microlitres of the enzyme extract was used for each assay. Final reagent concentrations were: 120mM Tricine, 50mM pyruvate, 10mM $MgCl_2$, 66mM FAD, 93μM thiamine pyrophosphate (TPP), pH 8, in the presence or absence of a herbicide of interest, in a volume of 750 μl. Incubations were carried out at 37°C for 30 minutes. Reactions were stopped with 250μl of 1.84M sulphuric acid, and decarboxylation of the acetolactate carried out at 37°C for 75 minutes, or at 60°C for 30 minutes. Assay blanks had sulphuric acid added prior to the enzyme extract. To the samples and blanks 650μl of α-naphthol in 2.73M NaOH/0.16%(w/v) creatine were added followed by incubation at 37°C for 30 minutes. After centrifugation at 30,000g the optical density of the supernatant was read at 540nm.

The assay procedure described was carried out on ALS enzyme extracted from each of the nine mutants for the following herbicides:

imazethapyr (PURSUIT)
imazapyr (ARSENAL)
imazaquin (SCEPTER)
chlorsulfuron (GLEAN)
chlorimuron (CLASSIC)
thiacarburon (HARMONY)

The enzyme activity graphs are given as Figures 3 to 8. In addition, homozygous lines of mutants 1 and 2 which were selected from generation $M_1BC_1S_2$, were also tested against the same group of herbicides and against representatives of the triazolopyrimidines and phenoxypyrimidines. The results are shown in Figures 9 to 16. The chemical structures of these herbicides are shown in Figure 2 of the accompanying drawings.

10. <u>ASSESSMENT OF INHIBITION</u>

From the enzyme assay data it is possible to derive, as a measure of the degree of inhibition the factor $ID_{50\%}$ for each mutant and for each herbicide. This factor is indicative of the herbicide concentration which gives 50% inhibition of the ALS activity in comparison with the wild-type control. It is also possible to derive the fold increase in resistance. These calculations are are given in Table 3 below.

TABLE 3

IMAZETHAPYR (heterozygous mutants)

Plant	ID50% µM	Fold Increase
Wild type	1.5	
Mutant 1	>1000	>660
2	230	153
3	2.5	2
5	125	83
6	60	40
7	4	3
8	>1000	>660
9	425	283
10	15	10

IMAZAPYR (heterozygous mutants)

Plant	ID50% µM	Fold Increase
Wild type	3	
Mutant 1	>1000	>333
2	540	180
3	4	1
5	300	100
6	5	2
7	10	3
8	>1000	>333
9	>1000	>333
10	35	12

IMAZAQUIN (heterozygous mutants)

Plant	ID50% µM	Fold Increase
Wild type	0.6	
Mutant 1	150	250
2	20	33
3	1.5	2
5	25	42
6	1.5	2
7	1	2
8	150	250
9	50	83
10	7	12

CHLORIMURON (heterozygous mutants)

Plant	ID50% nM	Fold Increase
Wild type	3.7	
Mutant 1	5	1
2	10	3
3	15	4
5	17	5
6	15	4
7	5	1
8	10	3
9	10	3
10	17	5

CHLORSULFURON (heterozygous mutants)

Plant	ID50% nM	Fold Increase
Wild type	18.5	
Mutant 1	65	4
2	65	4
3	35	2
5	40	2
6	65	4
7	8	1
8	75	4
9	50	3
10	35	2

THIACARBURON (heterozygous mutants)

Plant	ID50% nM	Fold Increase
Wild type	42.5	
Mutant 1	55	1
2	320	8
3	85	2
5	340	8
6	45	1
7	25	1
8	40	1
9	45	1
10	390	9

Similar calculation were made for homozygous mutants 1 and 2 only, the results being given in Table 4 below.

TABLE 4

MUTANT 1 (homozygous)

Herbicide	ID50% Wild	ID50% Mutant	Fold Increase
Imazapyr	3μM	>1mM	>333
Imazethapyr	1.5μM	750μM	500
Imazaquin	0.6μM	175μM	292
Chlorimuron	3.7nM	10nM	2.7
Chlorsulfuron	18.5nM	60nM	3.2
Thiacarburon	42.5nM	40nM	nil

MUTANT 2 (homozygous)

Herbicide	ID50% Wild	ID50% Mutant	Fold Increase
Imazapyr	3μM	675μM	225
Imazethapyr	1.5μM	400μM	267
Imazaquin	0.6μM	40μM	66
Chlorimuron	3.7nM	20nM	5.4
Chlorsulfuron	18.5nM	40nM	2.2
Thiacarburon	42.5nM	360nM	8.5
Triazolo-pyrimidine	0.07μM	0.12μM	1.7
Phenoxy-pyrimidine	0.03mM	0.45mM	22.5

11. <u>CROSS-RESISTANCE TESTING</u>

The seeds used in this screen were populations of mixed tolerant and sensitive seed, segregating 1:1, derived from each of the heterogygous, tolerant mutants crossed with homozygous, sensitive UE95. All of the tolerant progeny are heterozygous

for tolerance. Each of the mutants identified above were used except mutant 4. The controls were seed of self-pollinated UE95 which was pollinated in the same year as the tolerant lines.

One litre of compost was placed in each seed tray of dimensions 19cm x 11cm x 5cm deep and firmed down flat. Two furrows 1 cm deep were drawn in the surface of the compost in each tray and six seeds were sown in each furrow (total twelve seeds per tray). Some trays were sown with 24 seeds, in which case three furrows were made and 8 seeds sown per furrow.

In each test each tray contained either the seed of one mutant or of the UE95 control for each herbicide application rate and for the untreated control.

Five different types of ALS-inhibitor herbicides were tested on the mutants plus imazethapyr (PURSUIT) as a comparison. Four rates of each were applied; approximately 0.1x, 0.5x, 1x and 4x the estimated field rates for each herbicide. The rates are the same for mutant 3 to 10 but higher rates for chlorimuron (CLASSIC) and lower rates for chlorsulfuron (GLEAN) were applied to mutants 1 and 2.

The following herbicides, with their estimated field application rates were used:

chlorimuron (CLASSIC)	8 -13 g/Ha
chlorsulfuron (GLEAN)	4 -26 g/HA
imazaquin (SCEPTER)	100 -150 g/Ha
triazolopyrimidine	10 - 30 g/Ha
phenoxypyrimidine	100 - 200 g/Ha
imazethapyr (PURSUIT)	70 - 140 g/Ha

The rates of application used in the screening

tests were as follows:

chlorimuron (CLASSIC) 4,20,40,160 g/Ha (mutants 1 and 2); and, 8,40,80,320 g/Ha (mutants 3 to 10);
chlorsulfuron (GLEAN) 5,25,50,200 g/Ha (mutants 1 and 2); and, 1,5,10,40 g/Ha (mutants 3 to 10);
imazaquin (SCEPTER) 30,150,300,1200 g/Ha;
triazolopyrimidine 5,25,50,200 g/Ha;
phenoxypyrimidine 10,50,100,400 g/Ha; and,
imazethapyr (PURSUIT) 30,150,300,1200 g/Ha

The triazolopyrimidine and phenoxypyrimidine were formulated in an adjuvant wetting agent known as JF5969 and diluted with water to make a final concentration of 10% JF5969. The remaining compounds were diluted with water only.

The spray jet and parameters were as decribed above under "SCREENING". After spraying, the seeds were covered with 0.5 litre of compost and firmed down flat (2.5cm covering) and grown under the conditions described under "SCREENING" above.

Visual assessments were made of the plants after four weeks growth. Each plant was scored on a scale of zero to 5 and the height measured from soil surface to the tallest leaf tip. The scoring scale was as follows:

0 - 0 to 10% damage (little or no herbicidal effect)
1 - 11 to 25% damage
2 - 26 to 50% damage
3 - 51 to 85% damage
4 - 81 to 95% damage
5 - 96 to 100% damage (complete death of the plant)

In carrying out these assessments, plants which were obviously of the sensitive phenotype were ignored (approximately 50% of the plants).

Plants scoring 0, 1 or 2 were recorded and potted on into larger pots and grown to maturity.

The results are shown in Figures 17 to 22. Figure 23 shows a comparison of the results obtained as between heterozygous and homozygous plants.

12. INTERPRETATION OF THE RESULTS

The data generated for the heterozygotes by the enzyme assays and the dose response curves on the living plants, do not correlate precisely. To those skilled in the art, this will not be particularly unexpected. However, there is a general correlation in the sense that the general level of resistance shown in enzyme assays tends to be reflected in the glasshouse studies. Of course, the criteria for deciding what constitutes a useful mutant varies according to the herbicide, or spectrum of herbicides and the rate of application of the herbicide with which one is dealing and the relatively rich diversity in the spectrum of cross-resistance displayed by the mutants of this invention is seen as an advantage rather than an undesirable variation, allowing, as it does, selection of a mutant for the circumstances of intended use. For example, although a particular mutant may display relatively low resistance compared with the others against a particular herbicide this may make it eminently useful for providing plants which are intended to tolerate only small amounts of herbicide, for example, to resist a "carry-over" effect.

With this in mind, it is therefore possible to categorise the mutants on the basis of the enzyme assays and the glasshouse tests into "strong",

"intermediate", "weak" and "zero" resistance groups for each herbicide. This classification is summarised in Table 5 below.

TABLE 5

	MUTANT NUMBER	
IMAZETHAPYR	Enzyme Assay	Dose Response
Strong	1,8,9	1,5,8,9
Intermediate	2,5,10	2,6,10
Weak	3,6,7,	7,3
Zero	-	-
IMAZAPYR		
Strong	1,2,5,8,9,10	no data
Intermediate	6,7	no data
Weak	3	no data
Zero	-	no data
IMAZAQUIN	Enzyme Assay	Dose Response
Strong	1,8,9	1,2,8,9
Intermediate	2,5	5
Weak	3,6,7,10	3,6,10
Zero		7
CHLORSULFURON	Enzyme Assay	Dose Response
Strong	-	-
Intermediate	-,	3
Weak	1,2,3,5,6,8,9,10	5,7,9,10
Zero	7	1,2,6,8,9,10
CHLORIMURON	Enzyme Assay	Dose Response
Strong	-	-
Intermediate	10	-
Weak	1,2,3,5,6,7,9	3,10
Zero	8	1,2,5,6,7,~~8~~,9
THIACARBURON	Enzyme Assay	Dose Response
Strong	-	no data
Intermediate	2,5,10	no data
Weak	1,3,6,8,9	no data
Zero	7	no data

TABLE 5 (continued)

TRIAZOLOPYRIMIDINE	Enzyme Assay	Dose Response
Strong	no data	3
Intermediate	no data	1,2
Weak	no data	7,8,9
Zero	no data	5,6,10

PHENOXYPYRIMIDINE	Enzyme Assay	Dose Response
Strong	no data	–
Intermediate	no data	2,5,7
Weak	no data	3,8,9,10
	no data	1,6

13. PLANT BREEDING

The mutated lines of the present invention can be used in common with various second parent lines to produce herbicide resistant hybrids.

Material from the homozygous lines may be entered into a breeding programme involving further outcrossing, selfing, visual selection and herbicide screening in order to produce a range of new herbicide-tolerant hybrid seed.

The herbicide resistance trait can be transferred to new lines by the described mutation breeding approach or by conventional breeding practices, using selection for herbicide resistantce as described hereinabove. Biochemical and molecular screeing techniques are also available to those skilled in the art to aid the process. The use of genetic engineering techniques are readily conceivable to isolate and transfer the resistance gene.

The objective of a breeding preogramme may simply be the beneficial transfer of herbicide resistance or may be more complex, involving concurrent improvement of agronomic performance.

The foregoing represents the state of our work at the date of filing of the said International

Application. The description which now follows is relevant to the invention which is the subject of the present application.

14. Determination of the maize ALS wild-type gene sequence

A homologous maize gene probe was generated by PCR using data from the published literature (European Patent Application No. 257,993) which, it is to be noted contains only a partial sequence. The probe sequence was cloned into plasmid pIE103 using standard laboratory cloning procedures.

Two cDNA libraries, both constructed in λ-ZAP were screened with the insert from pIE103. These were (i) a pre-existing maize UE95 root cDNA library and (ii) a four-week old UE95 plant leaf cDNA library.

From an initial screening of 163,000 plaques using the pIE103 probe, twelve clones were plaque purified and *in vivo* excised to generate phagemids (plasmids). These wre designated R1, R2, R3, R4, R6, R9, R12, R17, R18, R19, R20 and R21. All were confirmed, by sequencing, to represent ALS genes, although most were truncated at an EcoR1 site internal to the coding region (see Figure ?): in fact only one, R21, contained coding sequence upstream of this restriction site.

R21 was fully sequenced by "oligo walking" using plasmid sequencing. The complete (>2kb) DNA sequence was thereby obtained.

15. Screening of the leaf library and sequencing of L3

Over one million plaques of a leaf DNA library were screened with pIE103 probe; only seven positives were recovered of which only three (L1,

L2, L3) hybridised to the ALS-9 probe. Sequencing of the 3'-non-translated regions of these clones indicated thqt each fell into one of two distinct classes which were designated Class 1 and Class 2.

All ALS clones analysed can be classified as either Class 1 or Class 2 on the basis of their 3' non-translated regions. Class 1 contains L1 and all the R clones mentioned above and Class 2 contains L2 and L3.

Confirmation of this work was given when European Patent Application Number 360,750 was published in which the complete genomic ALS sequences from a different maize line (known as Funk 2717) were given. Our Classes 1 and 2 corresponded very closely indeed to the classes C1 and C3 in the said application.

Clone L2 was found to be truncated, L3 was chosen as a representative of a UE95 wild type class 2 ALS gene for sequencing. The entire gene can be spanned by fourteen sequencing primers although some further sequencing was required to resolve ambiguities, particularly in the GC-rich 5'-region.

16. <u>Wild-type gene sequence comparisons</u>

A summary of maize ALS gene, Classes 1 and 2, characteristics is as follows:

Length of coding region:	1914 bp
Translation product:	638 amino acids
	~69Kd M
Homology:	approx 94% at DNA level in coding region but very reduced in 5' and 3'

	non-translated regions
UE95 vs Funk 2717 (Class 1 ALS sequences)	16bp differences in coding region resulting in four amino acid differences i.e. >99% homology

A comparison of the 3'non-translated regions of the UE95 Classes 1 and 2 type of genes showed that there is homology but the two genes are clearly distinguishable. Indeed, "class-specific" oligonucleotide probes can be designed based on the sequence os this region.

17. Determination of the mutant ALS gene sequences

Homozygous seed of Pursuit-resistant UE95 mutants 1 and 2, were sprayed with Pursuit and the leaf material harvested. cDNA libraries were constructed and screened with the ALS-9 probe. Two clones from mutant 1, and four clones from mutant 2 were plaque purified. All were Class 1 ALS genes.

Representative clones were selected for each line:

Mutant 1 = clone 1b
Mutant 2 = clone 2j
MGI line = clone Md

Each of these clones was sequenced with existing primers. Single base mutations were found in mutants 1 and 2 when the sequences were compared with the wild-type gene (R21) [see Figure 26].

18. Sequence of Mutant 1

A single base mutation was detected towards the 5' end of the coding region (indicated by an asterisk in Figure 24). This mutation, of guanine to adenine transition, results in an alanine to threonine substitution.

Such a mutation has been previously reported as conferring resistance to the sulphonylurea herbicides but our mutant 1 possesses no such resistance.

19. Sequence of Mutant 2

The sequence here showed a single base mutation from the wild-type. A guanine to adenine transition 52 bases from the 3' end of the coding region (indicated by an asterisk in Figure 25) results in a serine to asparagine substitution in the amino acid sequence.

CLAIMS

1. A DNA encoding a mutant acetolactate synthase and having the nucleotide sequence shown in Figure 24 or 25 of the drawingsherewith, and variations thereof permitted by the degeneracy of the genetic code.

2. A plant DNA having a nucleotide sequence having homology with that shown in Figure 1 and which contains a mutation at a position equivalent to number 171 in Figure 24 and/or number 1888 in Figure 25.

3. A plant DNA comprising the Apal to Stul or the Xhol to Stul restriction fragment shown in Figure 24 or the Xhol to NspHl restriction fragment shown in Figure 25, and variations in said DNAs as permitted by the degeneracy of the genetic code.

4. A plant which contains, stably integrated within its ~~genomes~~ genome by transformation, a DNA as claimed in any of claims 1 to 3.

5. Seeds of the plant claimed in claim 4.

6. Progeny of the plant claimed in claim 4 which are produced by crossing the said plant with another maize plant line.

7. A DNA coding for a mutant acetolactate synthase isolated from a seed deposited on 8th May 1989 with the National Collection of Industrial & Marine Bacteria, Aberdeen, United Kingdom, under the Accession Numbers NCIMB 40137 and 40136.

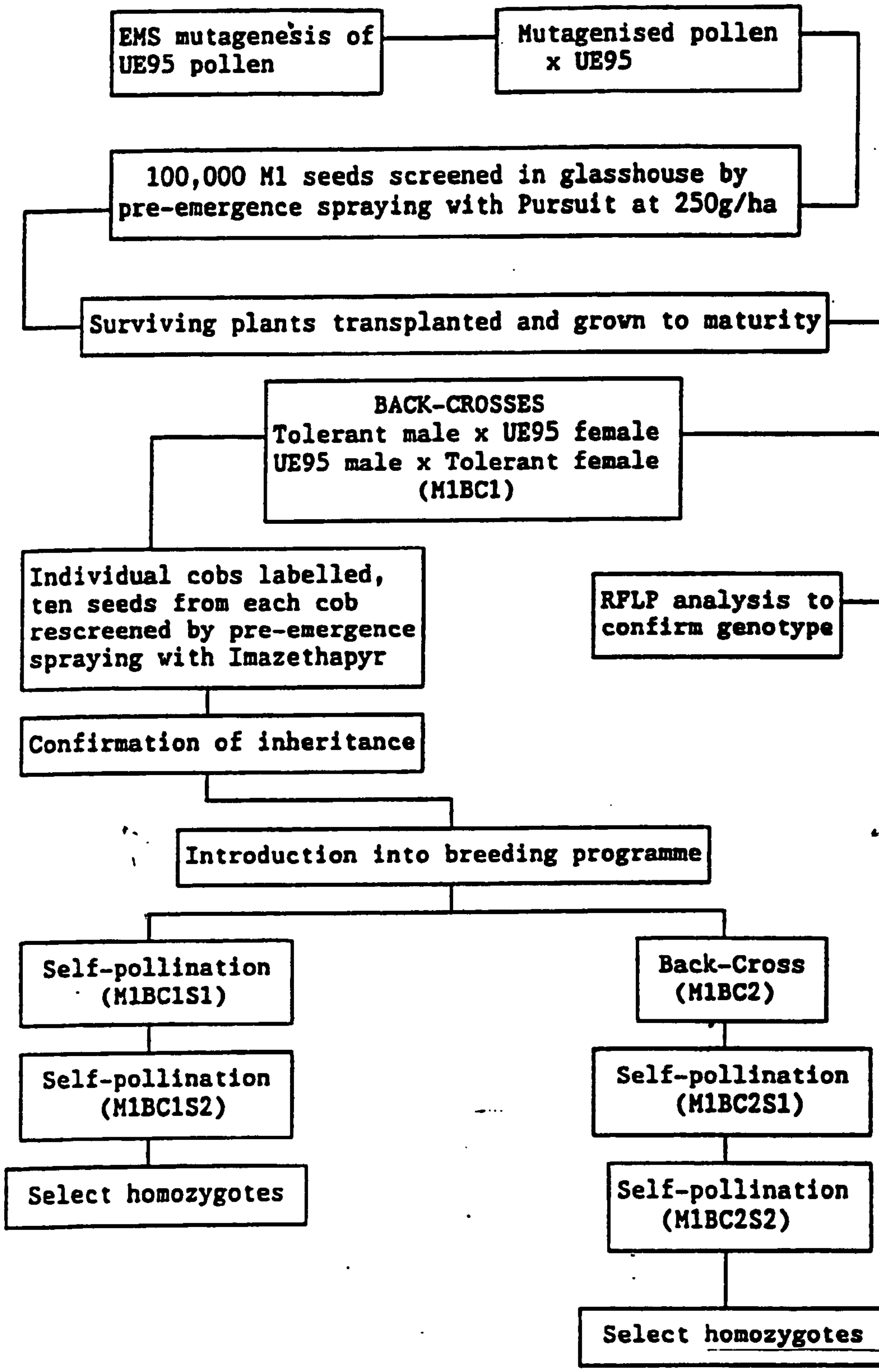
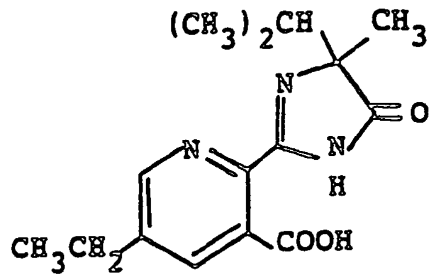
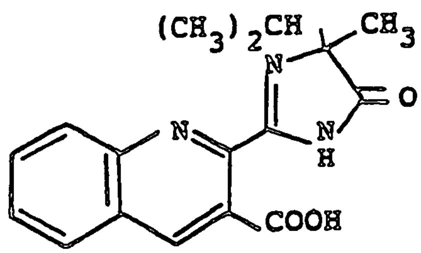

EMS mutagenesis of UE95 pollen
Mutagenised pollen x UE95
100,000 M1 seeds screened in glasshouse by pre-emergence spraying with Pursuit at 250g/ha
Surviving plants transplanted and grown to maturity
BACK-CROSSES
Tolerant male x UE95 female
UE95 male x Tolerant female
(M1BC1)
Individual cobs labelled, ten seeds from each cob rescreened by pre-emergence spraying with Imazethapyr
RFLP analysis to confirm genotype
Confirmation of inheritance
Introduction into breeding programme
Self-pollination (M1BC1S1)
Self-pollination (M1BC1S2)
Select homozygotes
Back-Cross (M1BC2)
Self-pollination (M1BC2S1)
Self-pollination (M1BC2S2)
Select homozygotes

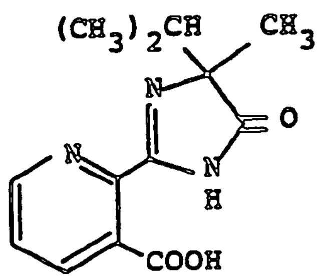
FIG. 1.



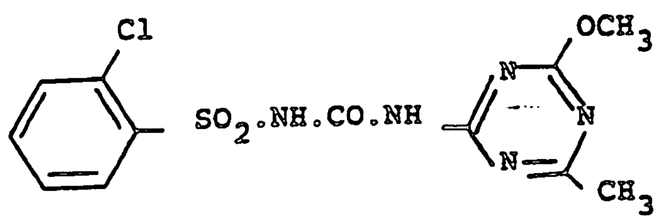
IMAZETHAPYR



IMAZAQUIN

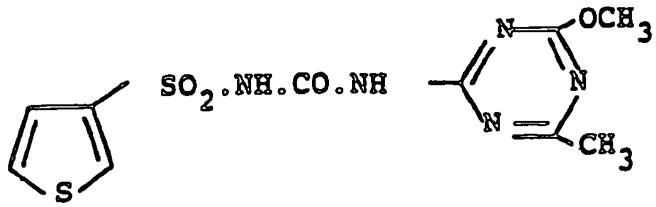


IMAZAPYR

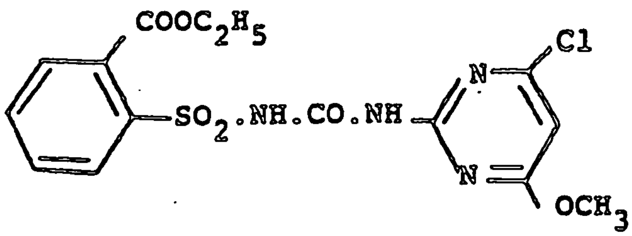


CHLORSULFURON

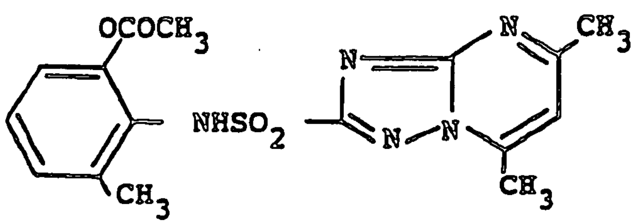
FIG. 2.



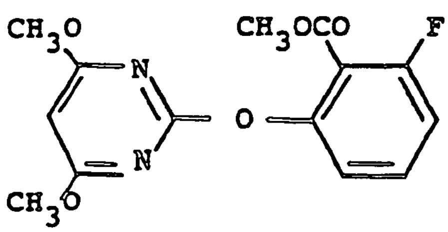
THIACARBURON



CHLORIMURON



TRIAZOLO-
PYRIMIDINE



PHENOXYPYRIMIDINE

FIG. 2 Continued

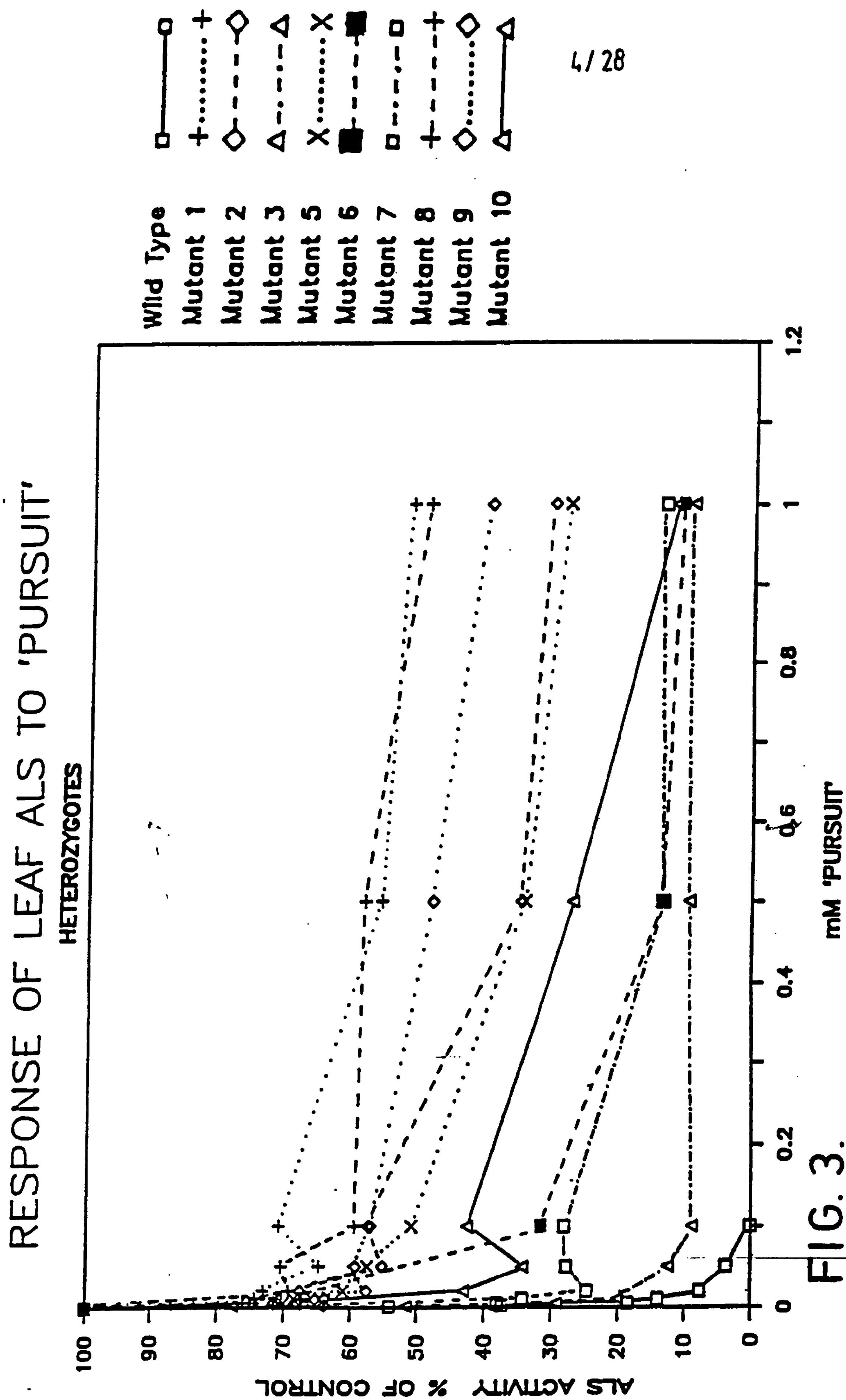

RESPONSE OF LEAF ALS TO 'PURSUIT'
HETEROZYGOTES
ALS ACTIVITY % OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
mM 'PURSUIT'
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10

FIG. 3.

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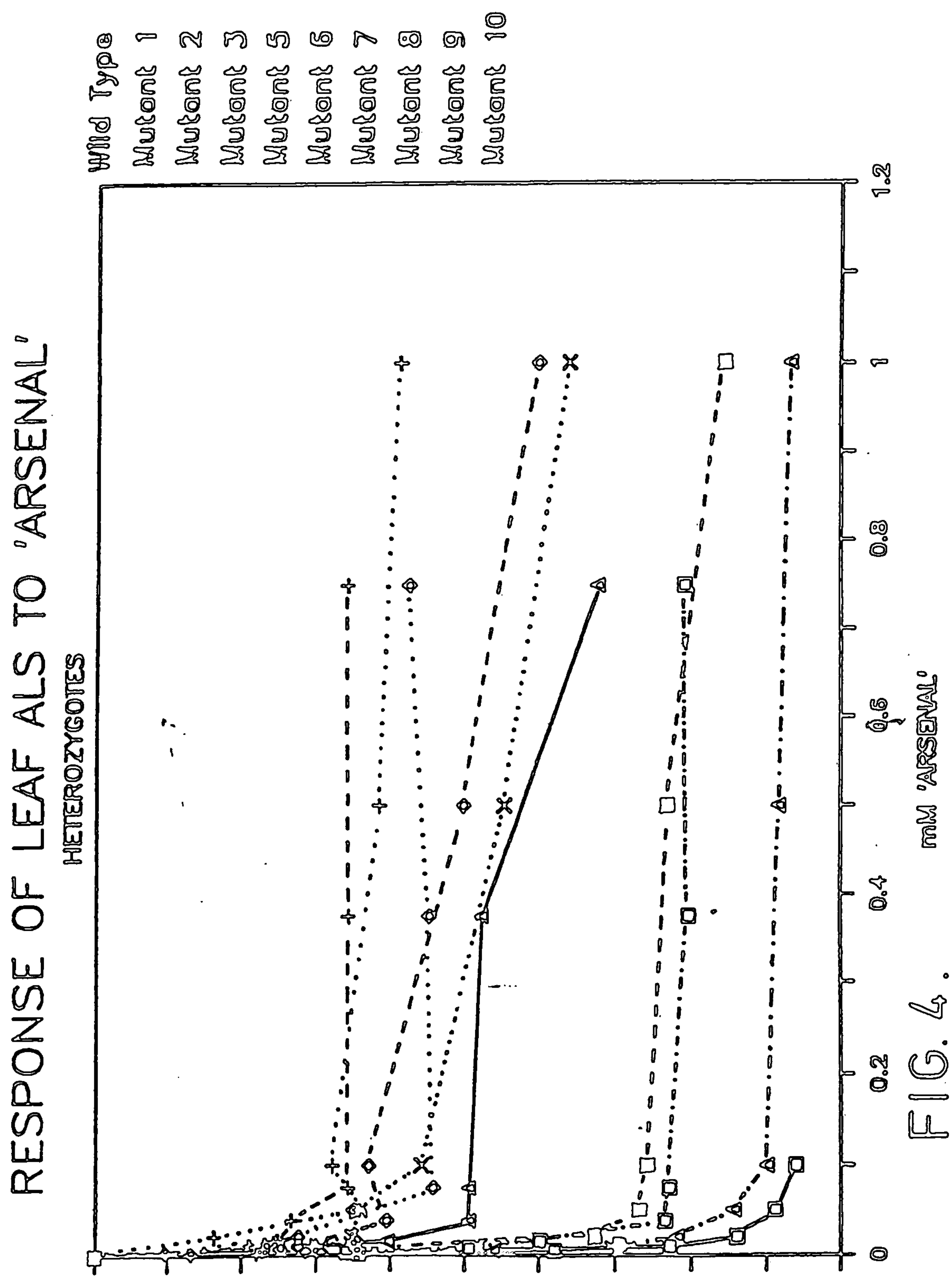
RESPONSE OF LEAF ALS TO 'ARSENAL'
HETEROZYGOTES
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10
ALS ACTIVITY % OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
mM 'ARSENAL'

FIG. 4.

RESPONSE OF LEAF ALS TO 'SCEPTER'

HETEROZYGOTES

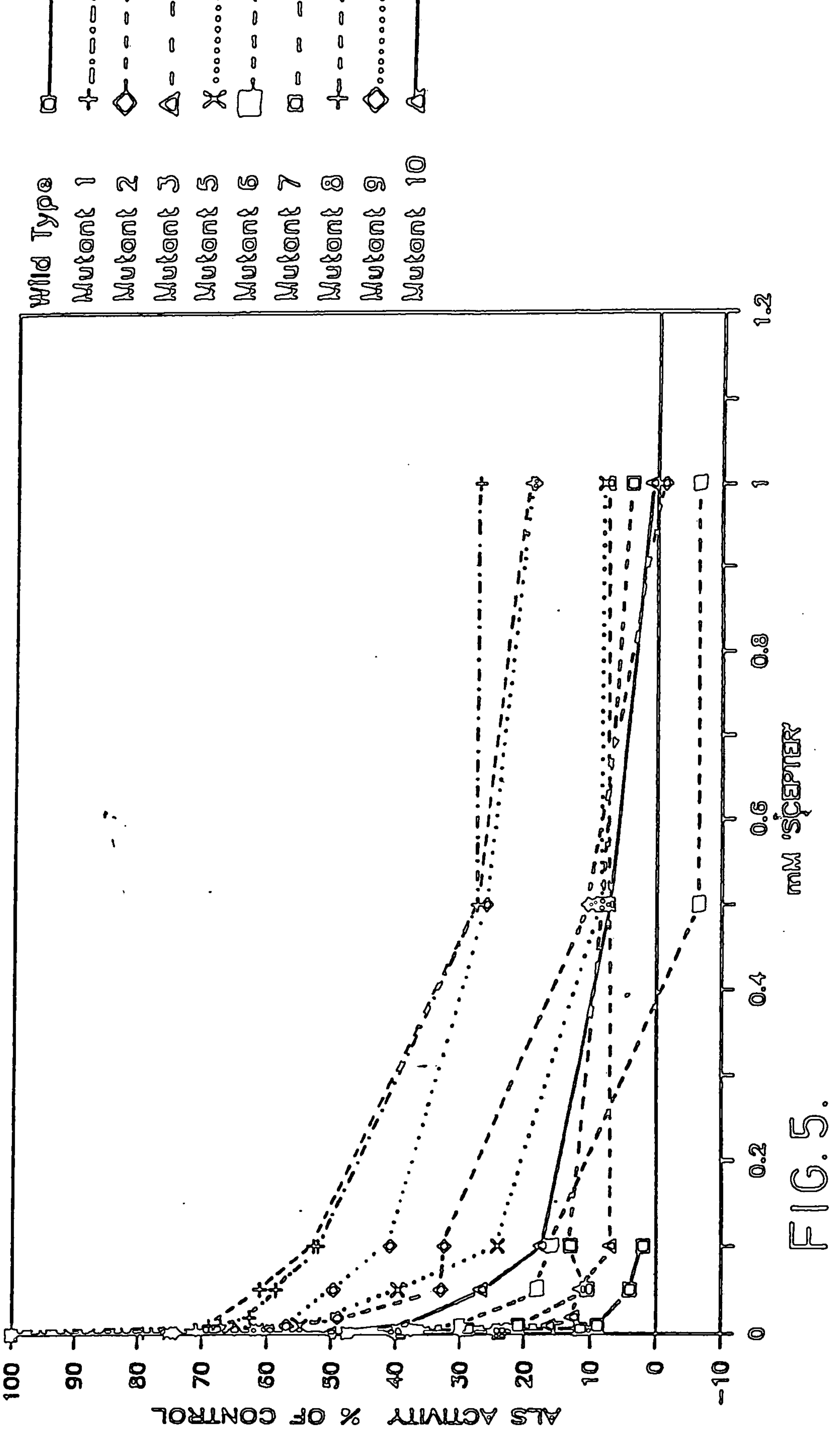

FIG. 5.

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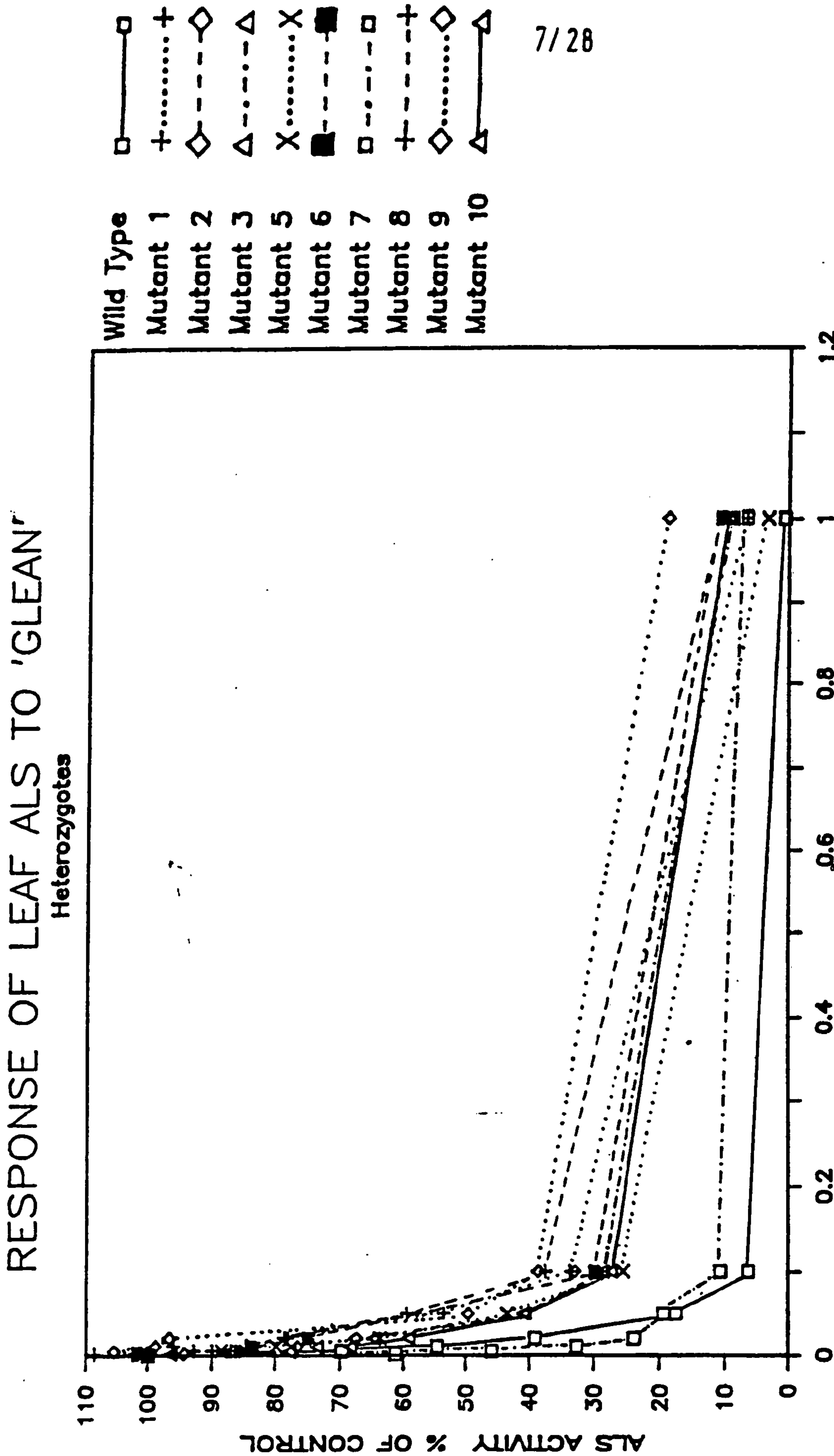


FIG.6.

SUBSTITUTE SHEET

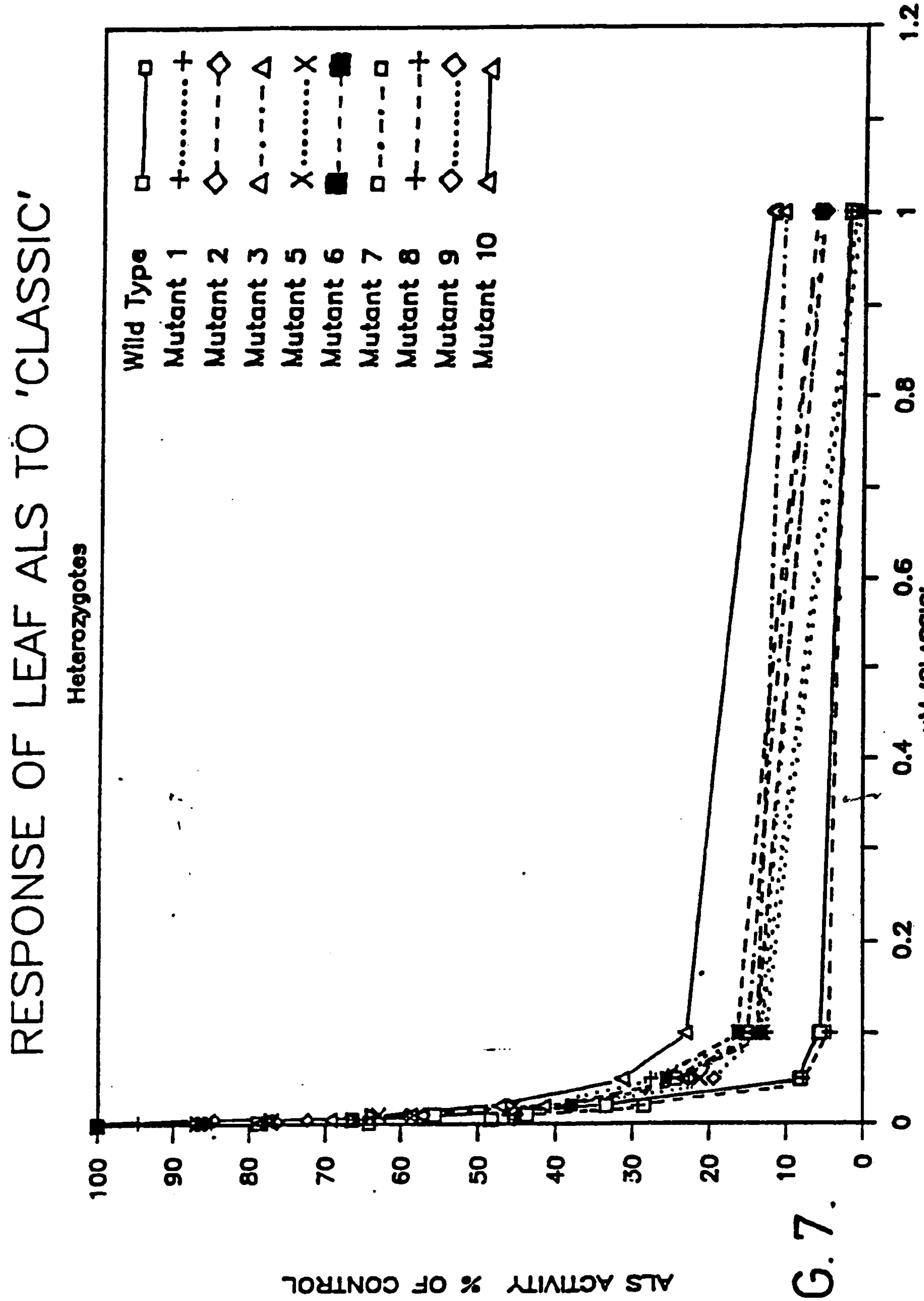

RESPONSE OF LEAF ALS TO 'CLASSIC'
Heterozygotes
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10
ALS ACTIVITY % OF CONTROL
uM 'CLASSIC'
0
0.2
0.4
0.6
0.8
1
1.2
100
90
80
70
60
50
40
30
20
10
0

FIG. 7.

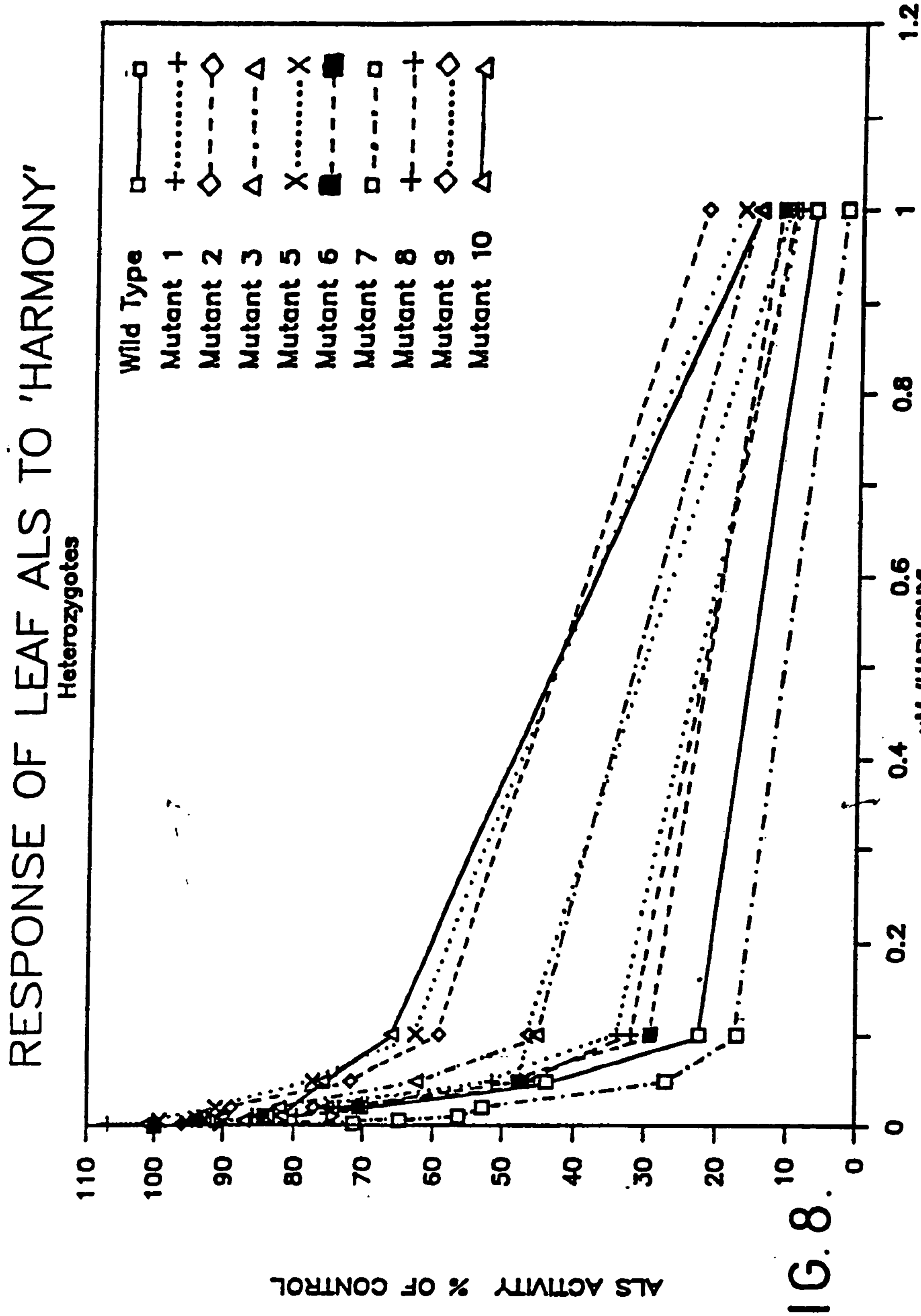

RESPONSE OF LEAF ALS TO 'HARMONY'
Heterozygotes
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10
110
100
90
80
70
60
50
40
30
20
10
0
ALS ACTIVITY % OF CONTROL
0
0.2
0.4
0.6
0.8
1
1.2
µM 'HARMONY'

FIG.8.

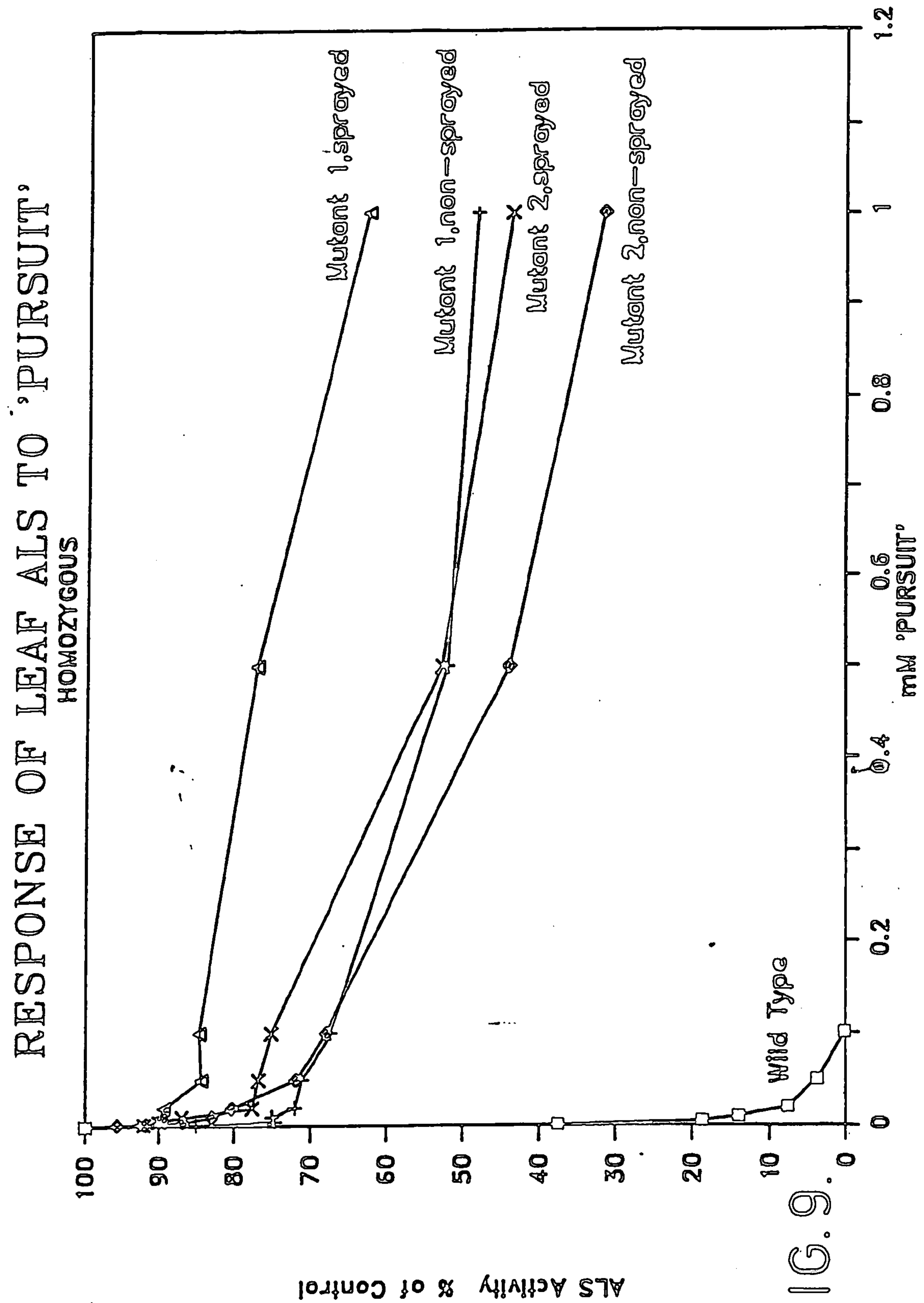

RESPONSE OF LEAF ALS TO 'PURSUIT'
HOMOZYGOUS
Mutant 1,sprayed
Mutant 1,non-sprayed
Mutant 2,sprayed
Mutant 2,non-sprayed
Wild Type
ALS Activity % of Control
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
mM 'PURSUIT'

FIG. 9.

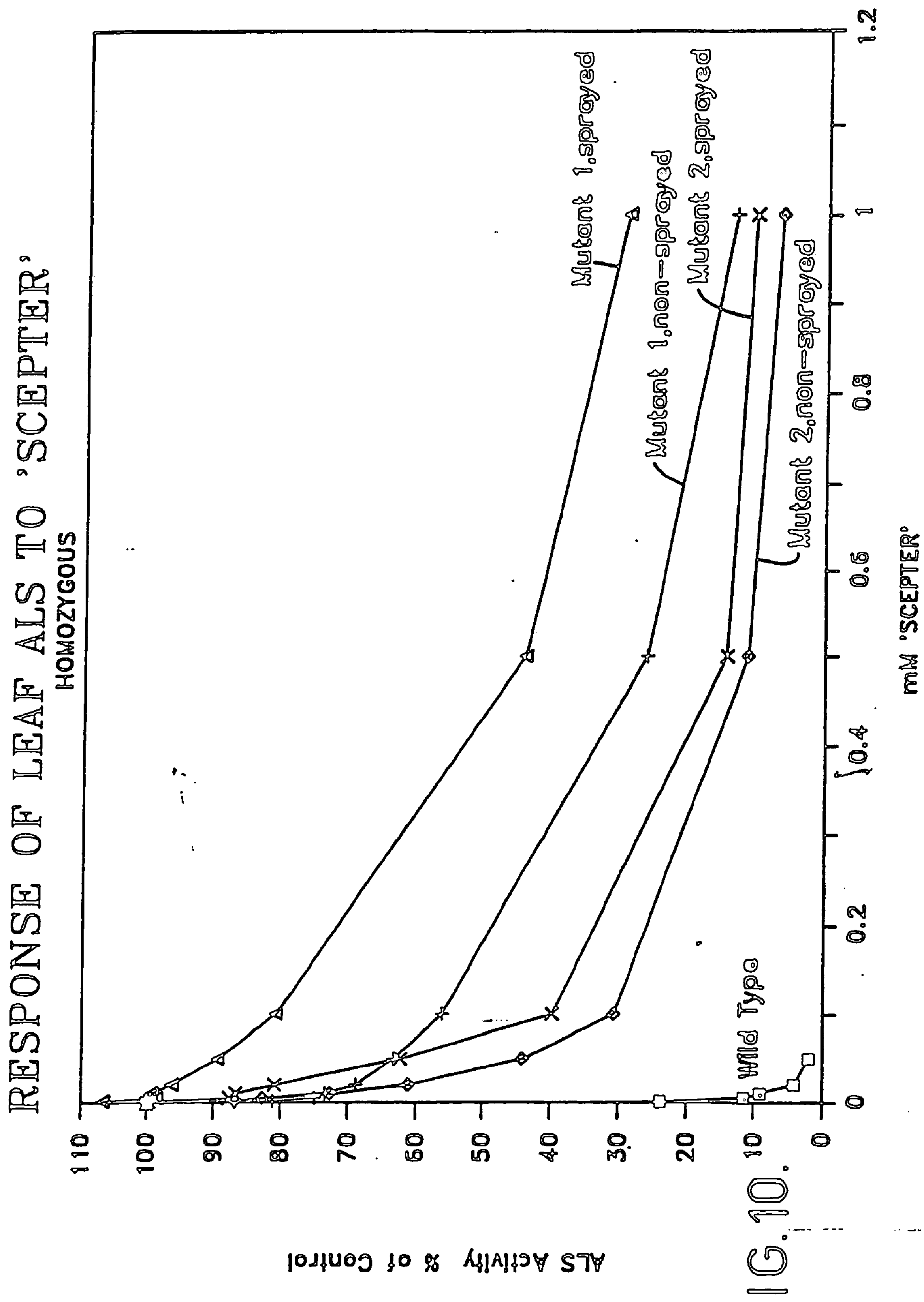

RESPONSE OF LEAF ALS TO 'SCEPTER'
HOMOZYGOUS
ALS Activity % of Control
mM 'SCEPTER'
Mutant 1,sprayed
Mutant 1,non-sprayed
Mutant 2,sprayed
Mutant 2,non-sprayed
Wild Type
0
10
20
30
40
50
60
70
80
90
100
110
0.2
0.4
0.6
0.8
1
1.2

FIG. 10.

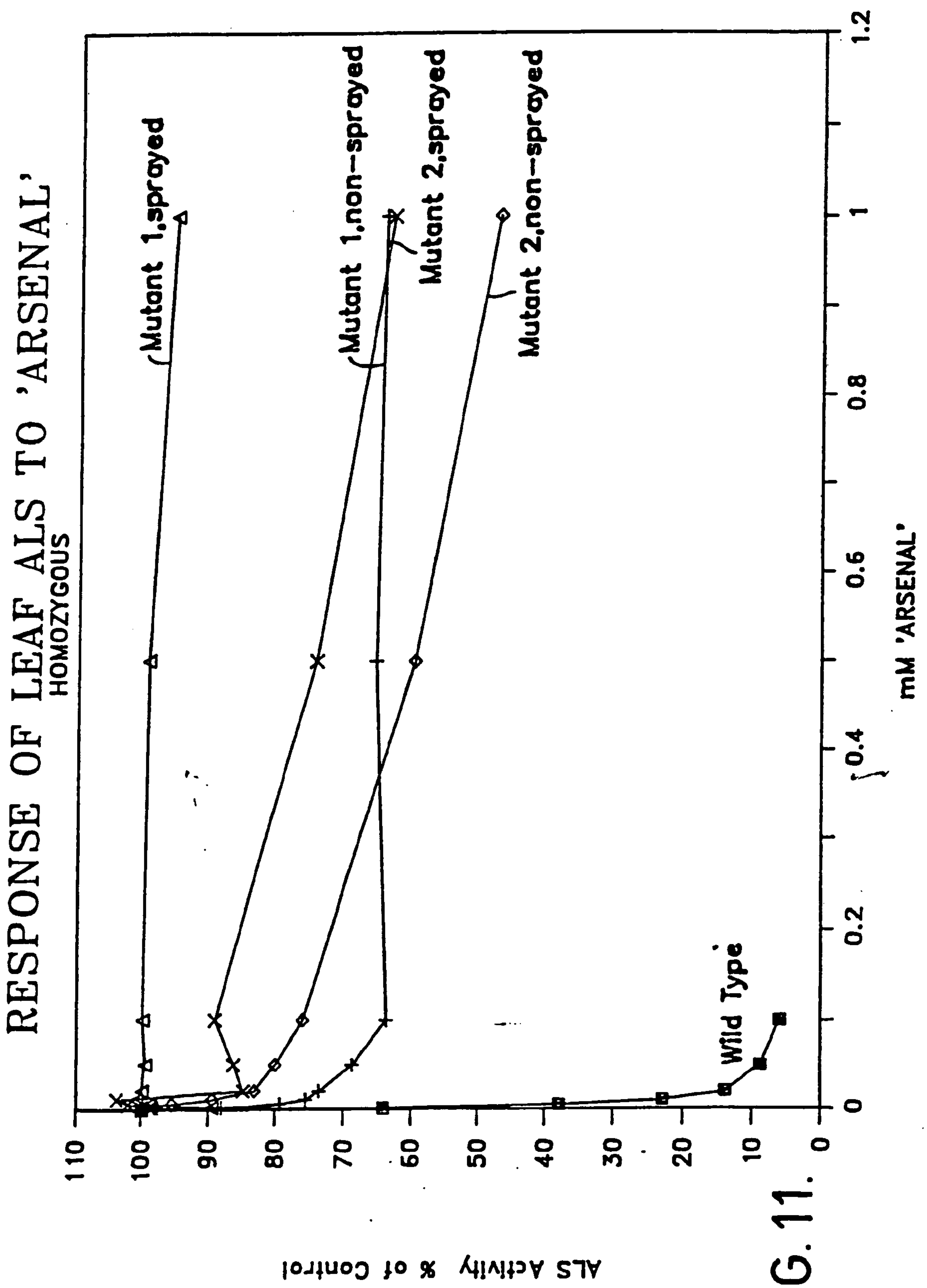

RESPONSE OF LEAF ALS TO 'ARSENAL'
HOMOZYGOUS
Mutant 1,sprayed
Mutant 1,non-sprayed
Mutant 2,sprayed
Mutant 2,non-sprayed
Wild Type
ALS Activity % of Control
mM 'ARSENAL'
110
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2

FIG. 11.

FIG. 12. RESPONSE OF LEAF ALS TO 'GLEAN'

HOMOZYGOUS, NON-SPRAYED

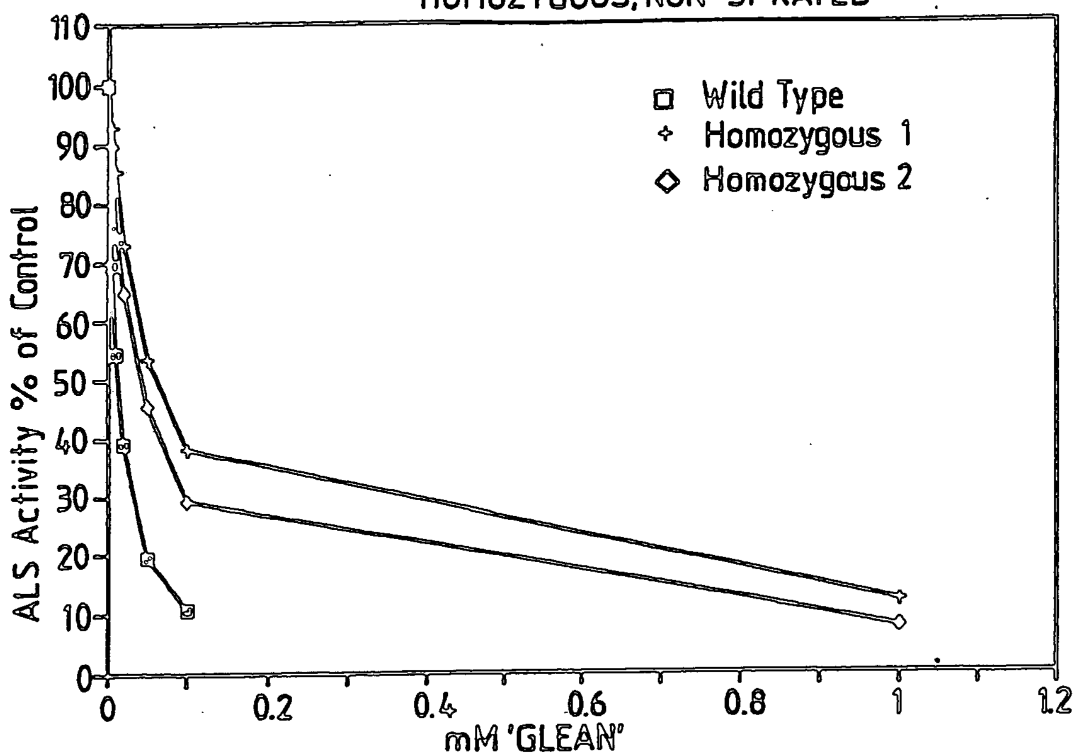


FIG. 13. RESPONSE OF LEAF ALS TO 'CLASSIC'

HOMOZYGOUS, NON-SPRAYED

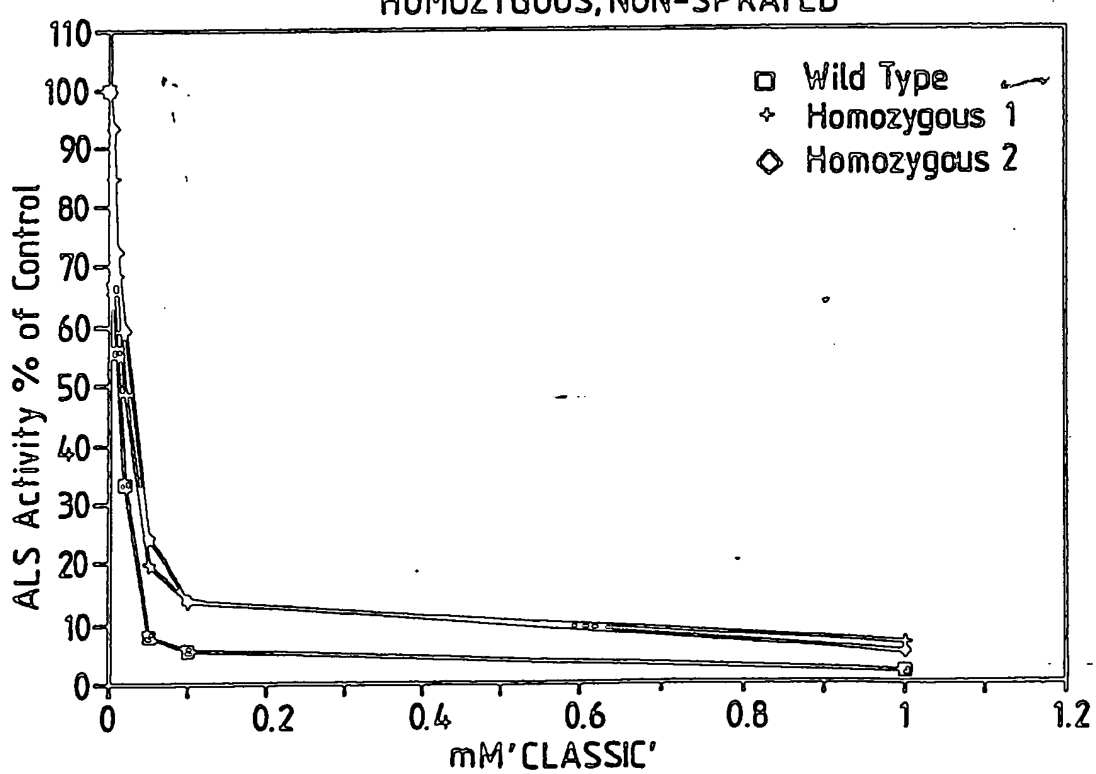


FIG. 14.

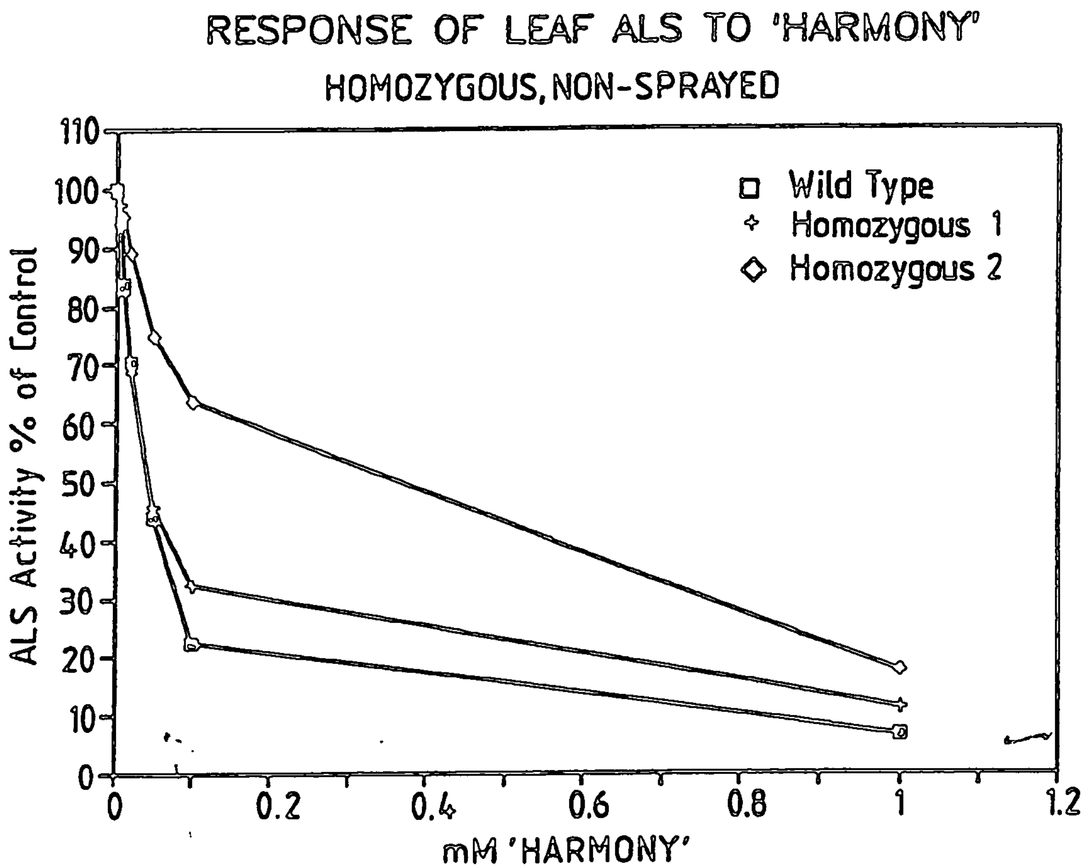

RESPONSE OF LEAF ALS TO 'HARMONY'
HOMOZYGOUS, NON-SPRAYED
Wild Type
Homozygous 1
Homozygous 2
ALS Activity % of Control
110
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
mM 'HARMONY'

FIG. 15. RESPONSE OF ALS TO A PHENOXYPYRIMIDINE

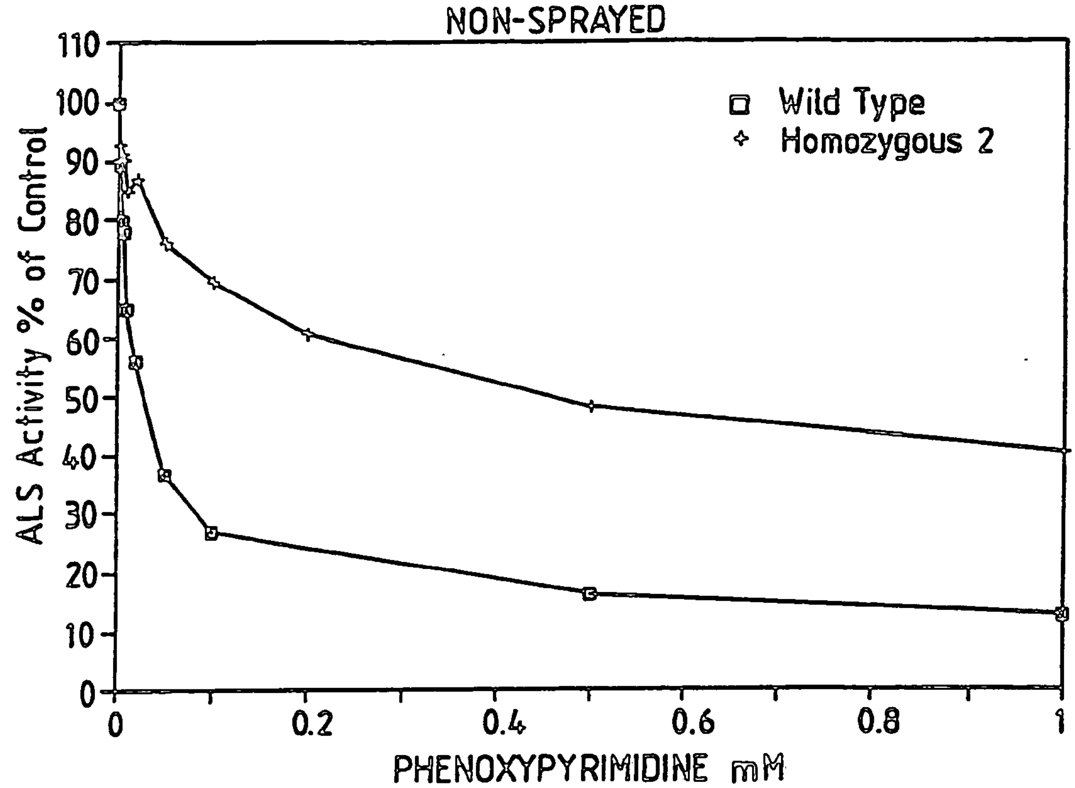


FIG. 16. RESPONSE OF ALS TO A TRIAZOLOPYRIMIDINE

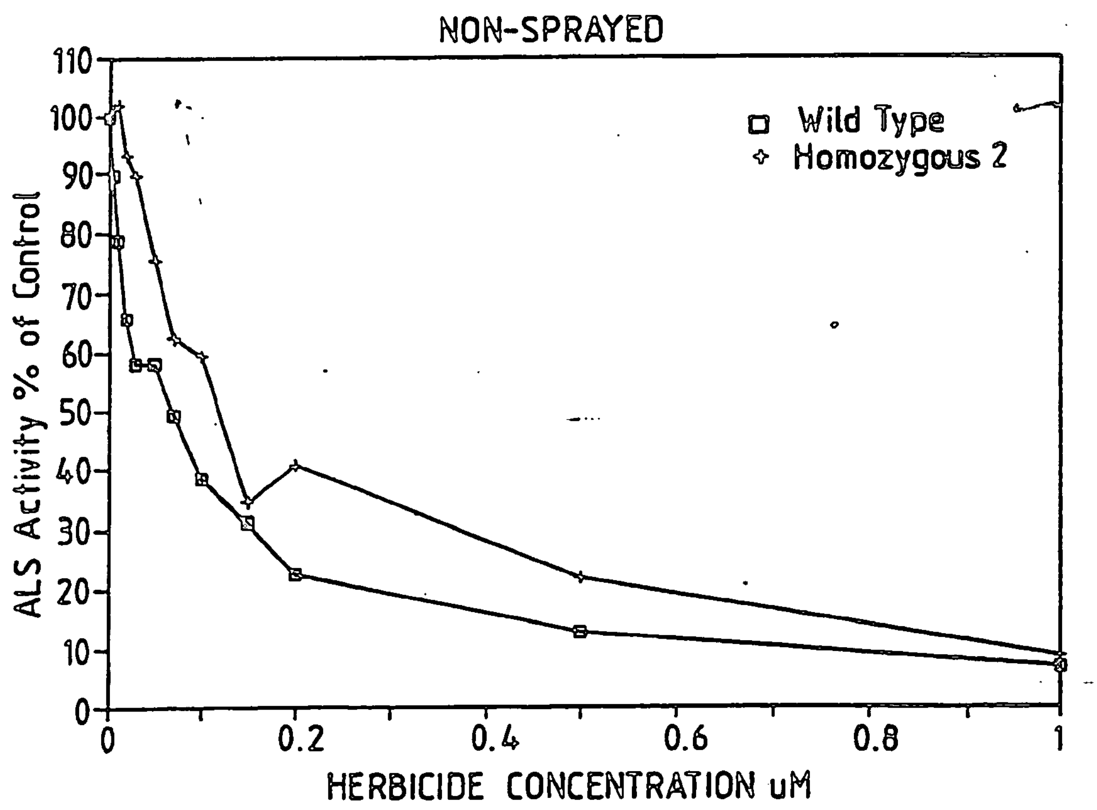
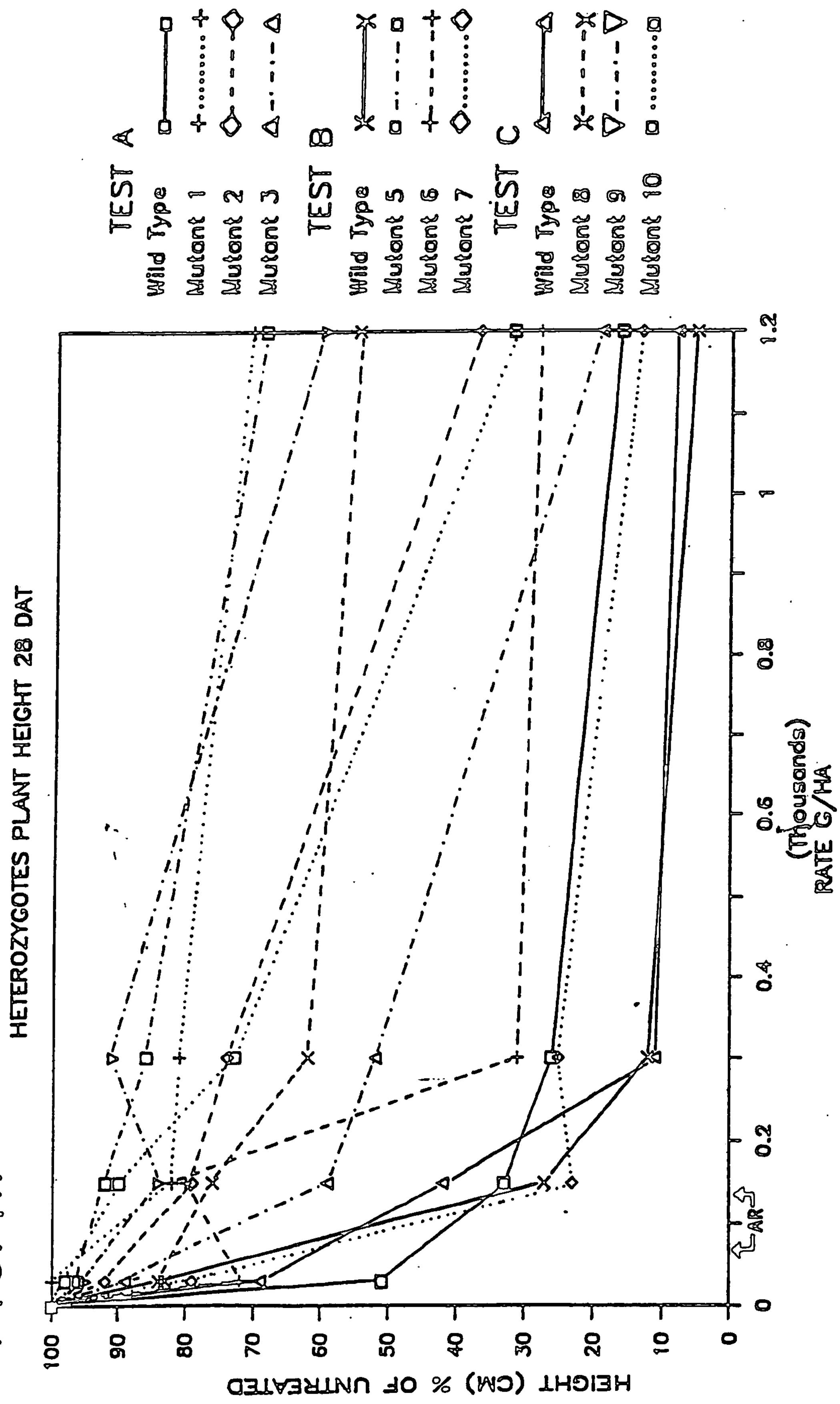


FIG. 17. PURSUIT DOSE RESPONSE



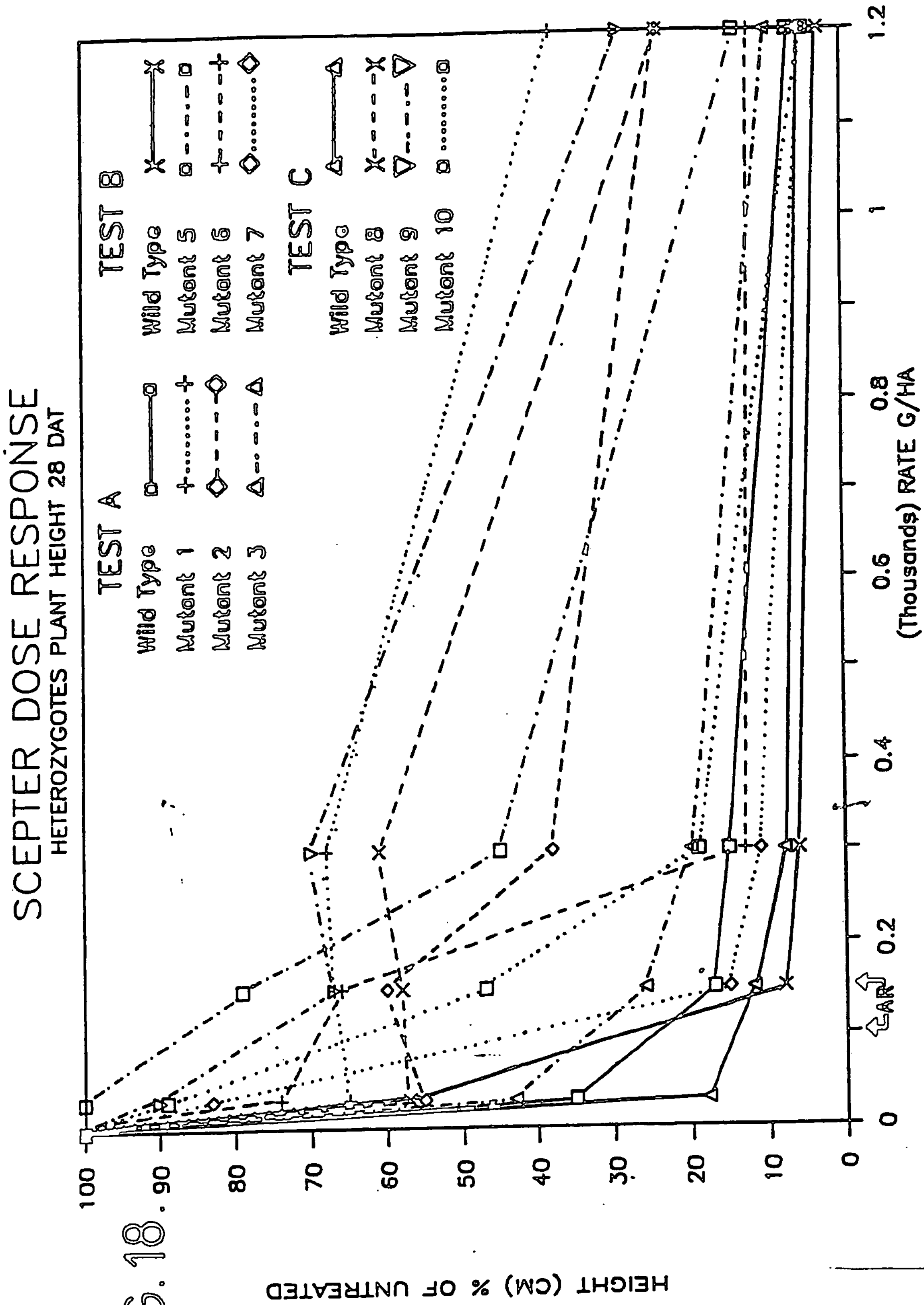

SCEPTER DOSE RESPONSE
HETEROZYGOTES PLANT HEIGHT 28 DAT
TEST A
Wild Type
Mutant 1
Mutant 2
Mutant 3
TEST B
Wild Type
Mutant 5
Mutant 6
Mutant 7
TEST C
Wild Type
Mutant 8
Mutant 9
Mutant 10
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
AR
(Thousands) RATE G/HA
HEIGHT (CM) % OF UNTREATED

FIG. 18

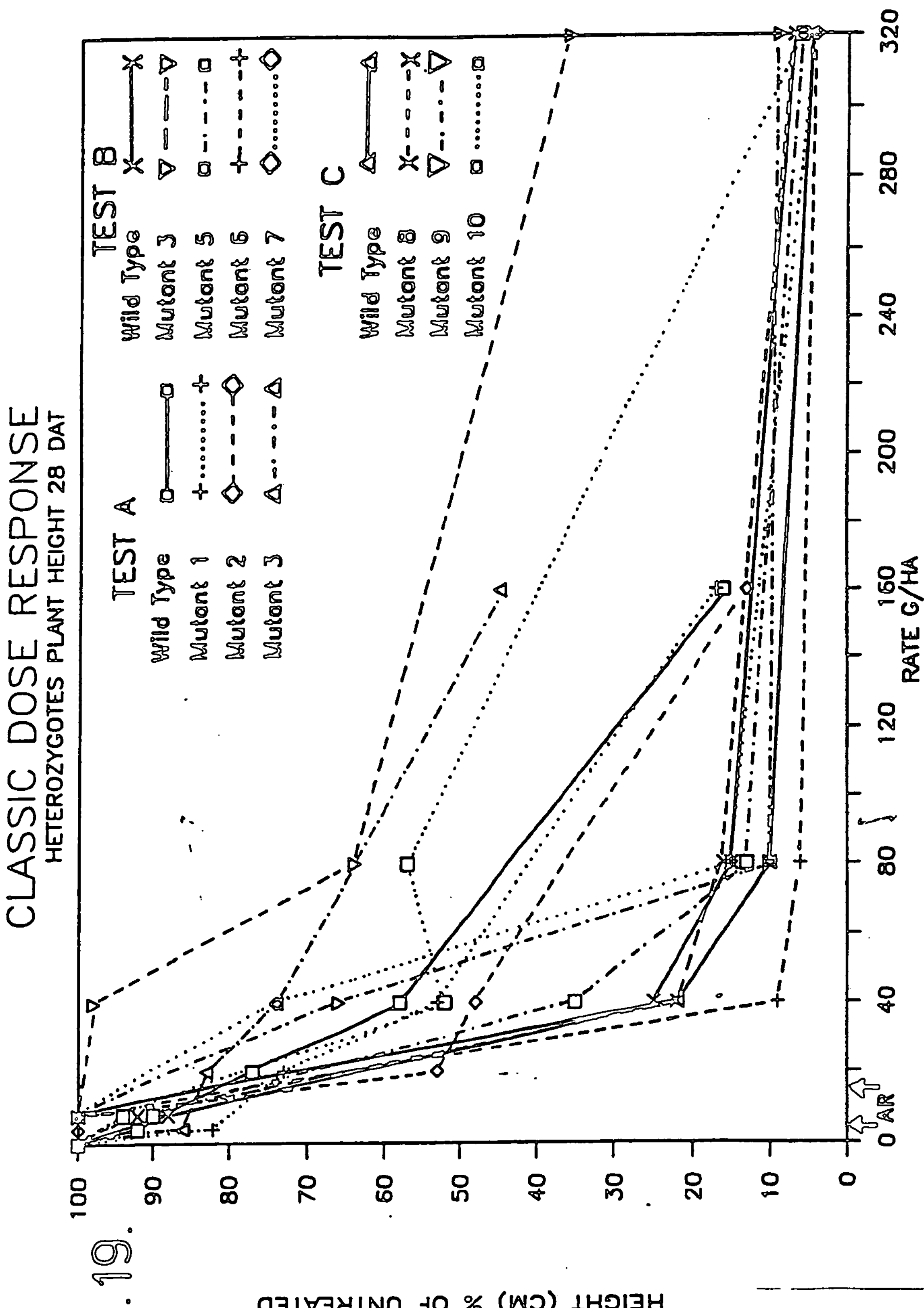
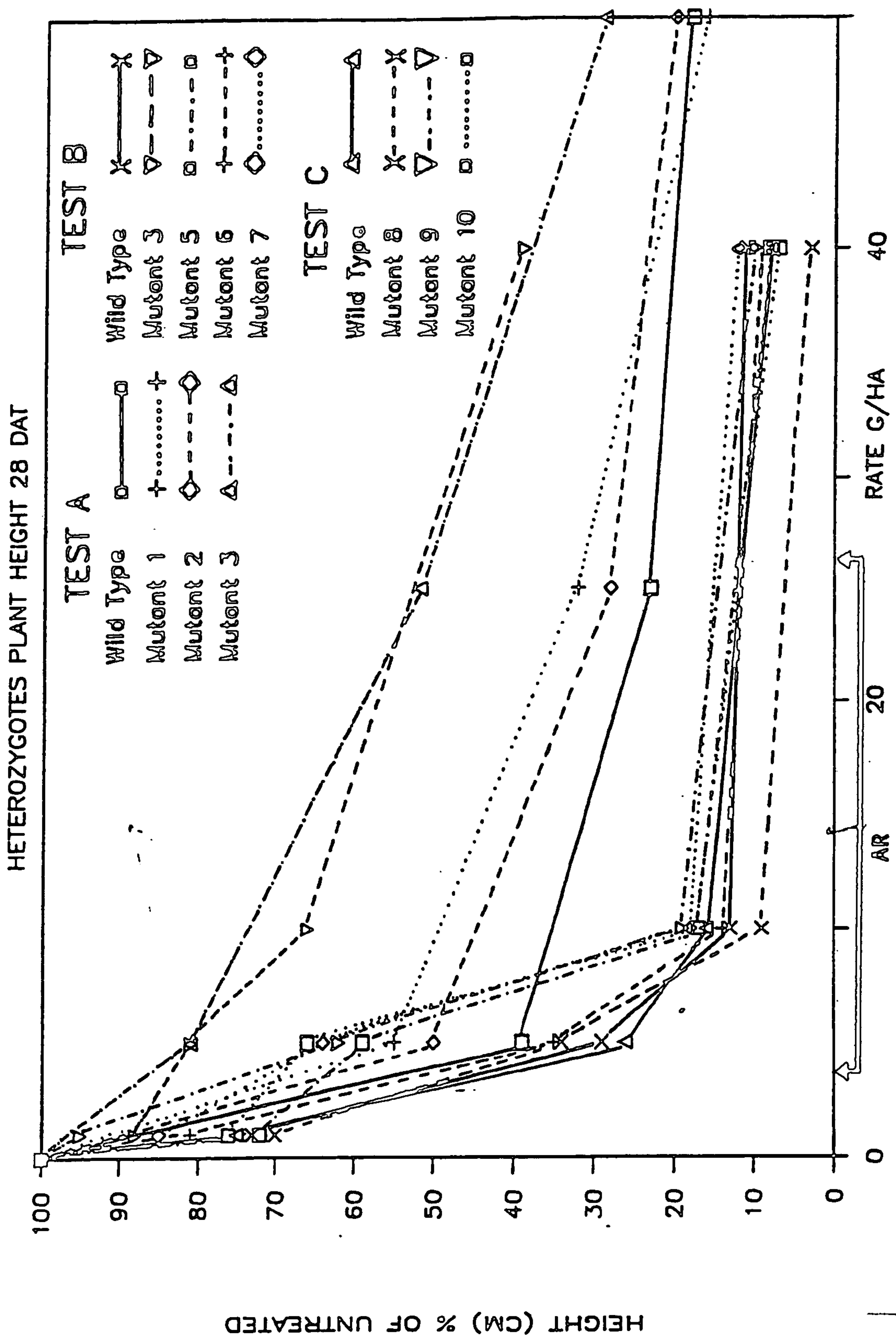

CLASSIC DOSE RESPONSE
HETEROZYGOTES PLANT HEIGHT 28 DAT
TEST A
Wild Type
Mutant 1
Mutant 2
Mutant 3
TEST B
Wild Type
Mutant 3
Mutant 5
Mutant 6
Mutant 7
TEST C
Wild Type
Mutant 8
Mutant 9
Mutant 10
HEIGHT (CM) % OF UNTREATED
RATE G/HA
AR
0
40
80
120
160
200
240
280
320
100
90
80
70
60
50
40
30
20
10
0

FIG.19.

FIG. 20. GLEAN DOSE RESPONSE



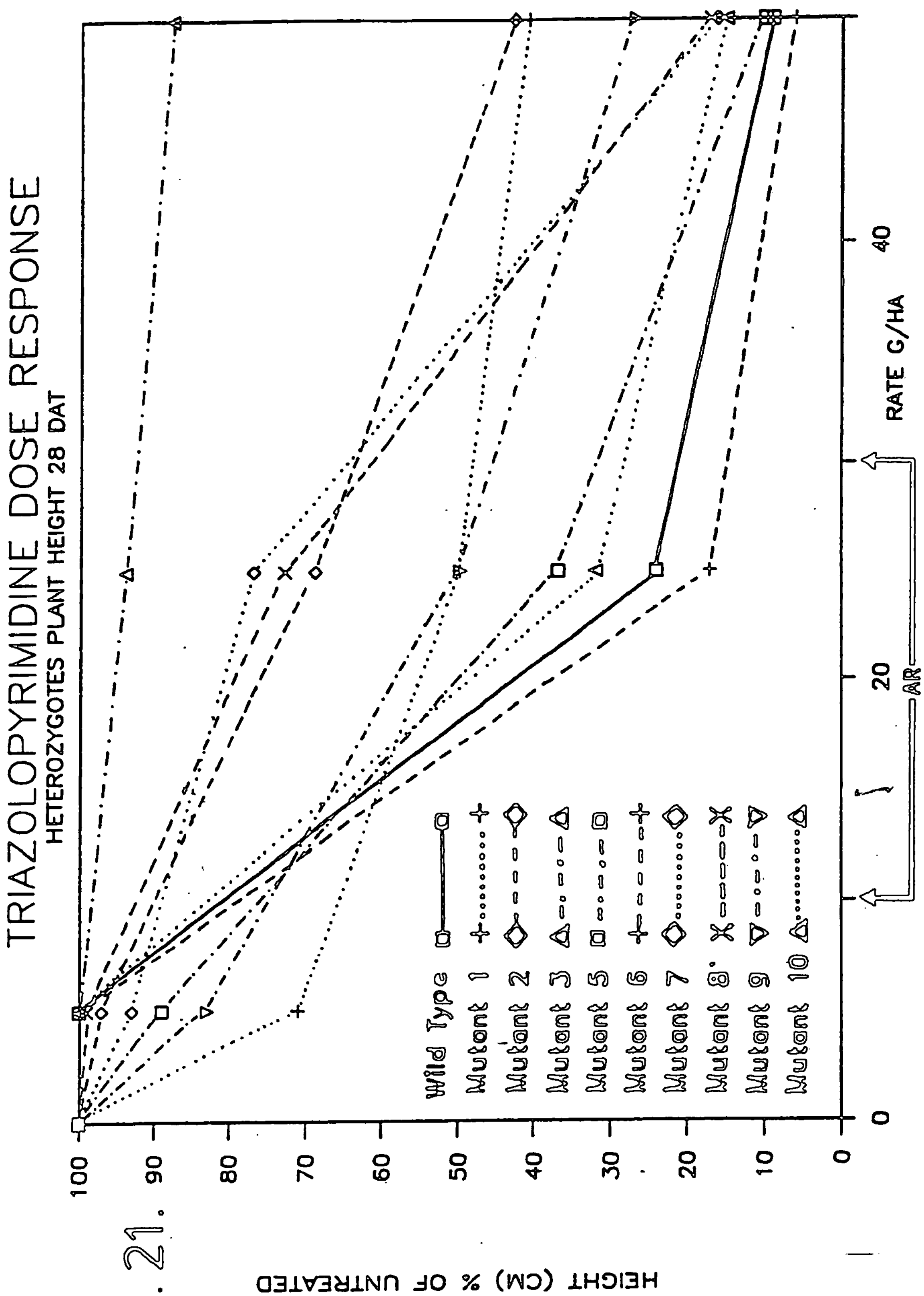

TRIAZOLOPYRIMIDINE DOSE RESPONSE
HETEROZYGOTES PLANT HEIGHT 28 DAT
HEIGHT (CM) % OF UNTREATED
100
90
80
70
60
50
40
30
20
10
0
0
20
40
RATE G/HA
AR
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10

FIG. 21.

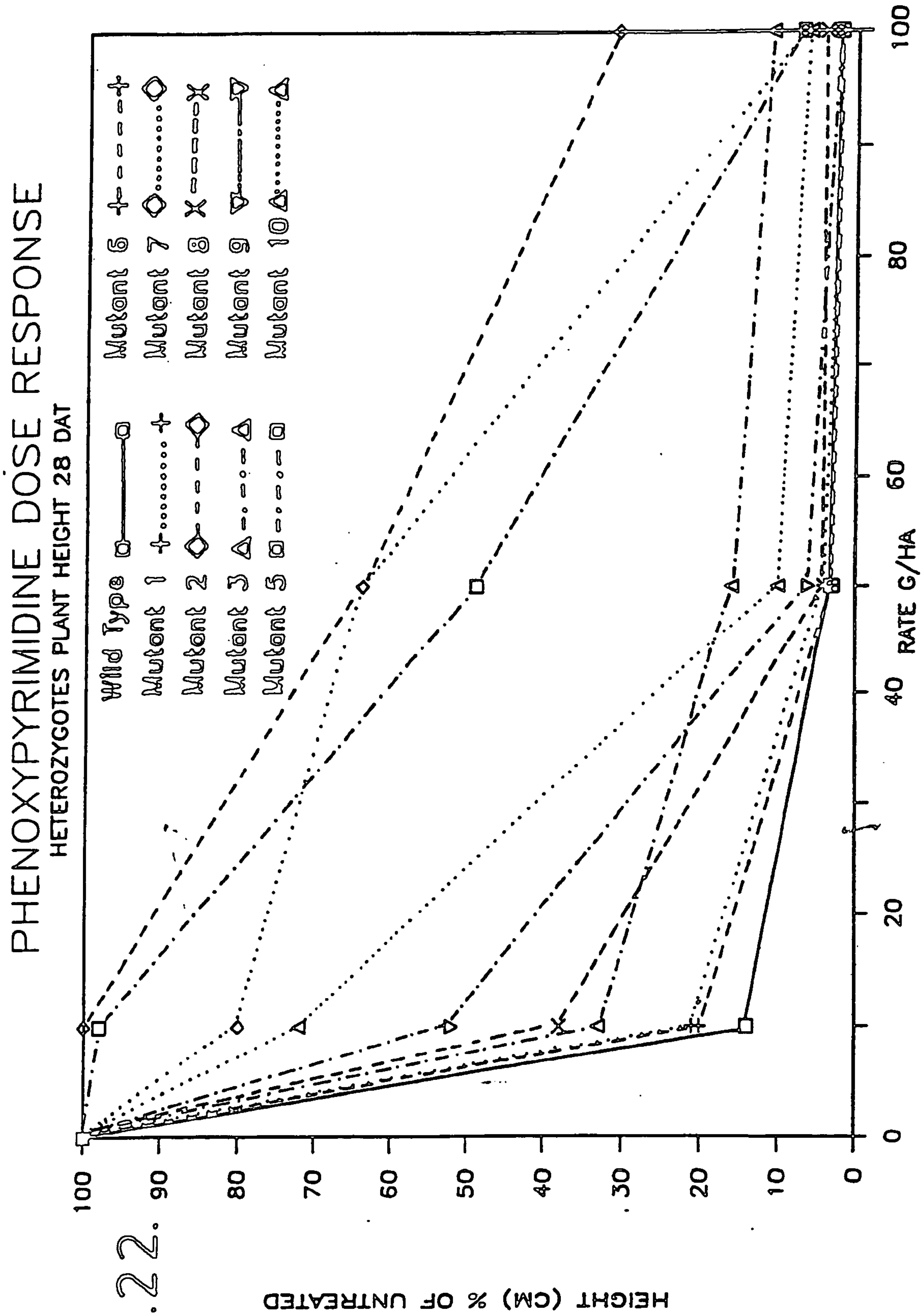

PHENOXYPYRIMIDINE DOSE RESPONSE
HETEROZYGOTES PLANT HEIGHT 28 DAT
Wild Type
Mutant 1
Mutant 2
Mutant 3
Mutant 5
Mutant 6
Mutant 7
Mutant 8
Mutant 9
Mutant 10
RATE G/HA
HEIGHT (CM) % OF UNTREATED
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
100

FIG.22.

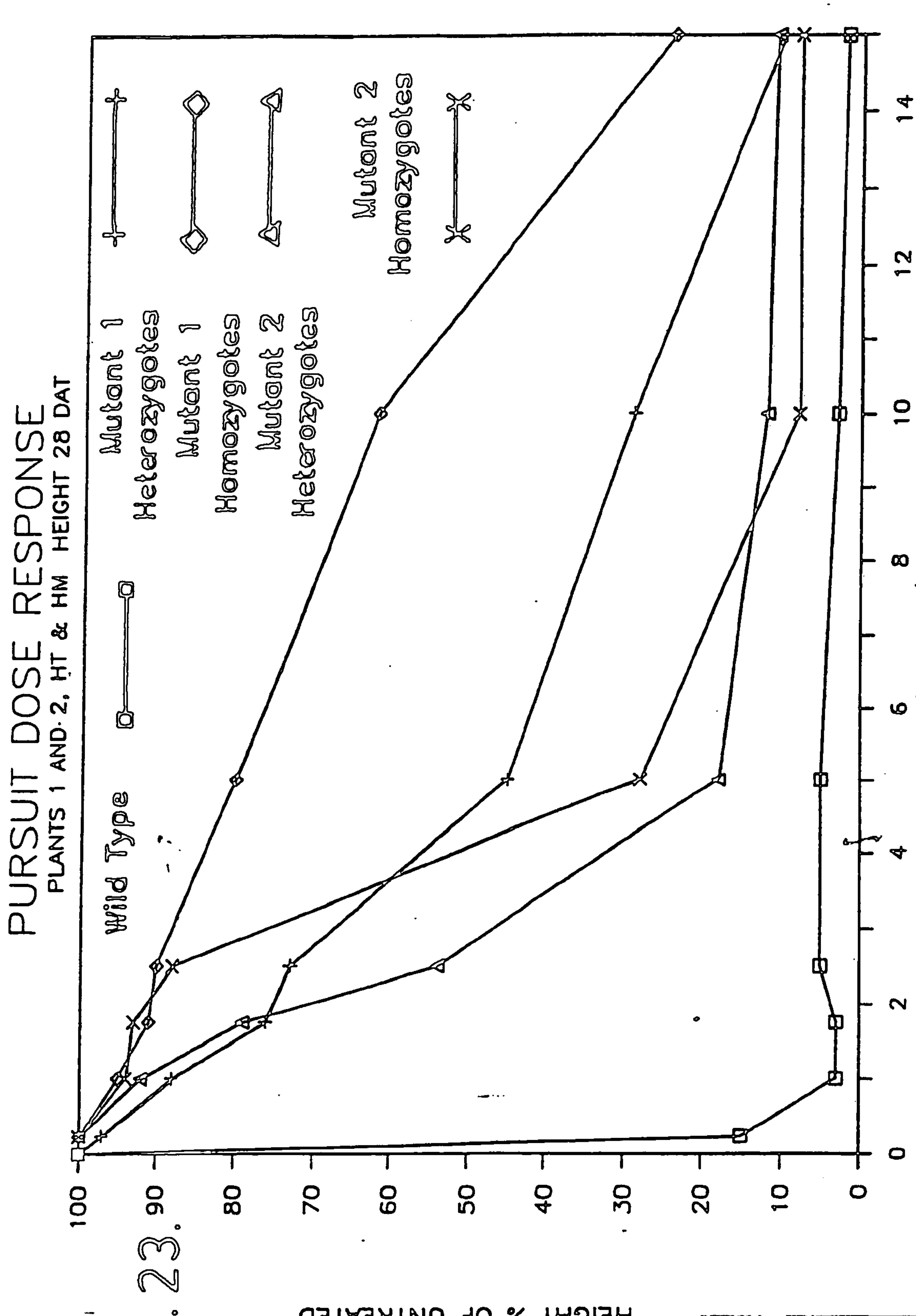

PURSUIT DOSE RESPONSE
PLANTS 1 AND 2, HT & HM HEIGHT 28 DAT
Wild Type
Mutant 1 Heterozygotes
Mutant 1 Homozygotes
Mutant 2 Heterozygotes
Mutant 2 Homozygotes
HEIGHT % OF UNTREATED
RATE KG/HA
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14

FIG. 23.

FIG. 24

```
CCCTCGCNGN GCCCATCAGG TGCTCAGCGC GTCACCCGCC ATCGCGATGG CTCCCCCGGC
CACCCCGCTC CGGCCGTGGG GCCCCACCGA GCCCCGCAAG GGTGCTGACA TCCTCGTCGA
GTCCCTCGAG CGCTGCGGCG TCCGCGACGT CTTCGCCTAC CCCGGCGGCA CGTCCATGGA
GATCCACCAG GCACTCACCC GCTCCCCCGT CATCGCCAAC CACCTCTTCC GCCACGAGCA
AGGGGAGGCC TTTGCGCTCC GCGTACGCGC GCTCCTCGGC GCGCGTCGGC GTCTGCATCG
CCACCTCCGG CCCCGGCGCC ACCAACCTAG TCTCCGCGCT CGCCGACGCN CTGCTCGATT
CCGTCCCCAT GGTCGCCATC ACGGGACAGG TGCCGCGACG CATGATTGGC ACCGACGCCT
TCCAGGAGAC GCCCATCGTC GAGGTCACCC GCTCCATCAC CAAGCACAAC TACCTGGTCC
TCGACGTCGA CGACATCCCC CGCGTCGTGC AGGAGGCTTT CTTCCTCGCC TCCTCTGGTC
GACCAGGGCC GGTGCTTGTC GACATCCCCA AGGACATCCA GCAGCAGATG GCGGTGCCTG
TCTGGGACAA GCCCATGAGT CTGCCTGGGT ACATTGCGCG CCTTCCCAAG CCCCCTGCGA
CTGAGTTGCT TGAGCAGGTG CTGCGTCTTG TTGGTGAATC GCGGCGCCCT GTTCTTTATG
TGGGCGGTGG CTGCGCACGA TCTGGTGAGG AGTTGCGACG CTTTGTGGAG CTGACTGGAA
TCCCGGTCAC AACTACTCTT ATGGGCCTCG GCAACTTCCC CAGCGACGAC CCACTGTCTC
TGCGCATGCT TGGTATGCAT GGCACGGTGT ATGCAAATTA TGCAGTGGAT AAGGCCGATC
TGTTGCTTGC ATTTGGTGTG CGGTTTGATG ATCGCGTGAC AGGGAAGATT GAGGCTTTTG
CAAGCAGGGC TAAGATTGTG CACGTTGATA TTGATCCCGC TGAGATTGGC AAGAACAAGC
AGCCACATGT GTCCATCTGT GCAGATGTTA AGCTTGCTTT GCAGGGCATG AATGCTCTTC
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FIG.24 cont.

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TTGAAGGAAG CACATCAAAG AAGAGCTTTG ACTTTGGCTC ATGGAACGAT GAGTTGGATC
AGCAGAAGAG GGAATTCCCC CTTGGGTATA AAACATCTAA TGAGGAGATC CAGCCACAAT
ATGCTATTCA GGTTCTTGAT GAGCTGACGA AAGGCGAGGC CATCATCGGC ACAGGTGTTG
GGCAGCACCA GATGTGGGCG GCACAGTACT ACACTTACAA GCGGCCAAGG CAGTGGTTGT
CTTCAGCTGG TCTTGGGGCT ATGGGATTTG GTTTGCCGGC TGCTGCTGGT GCTTCTGTGG
CAAACCCAGG TGTCACTGTT GTTGACATCG ATGGAGATGG TAGCTTTCTC ATGAACGTTC
AGGAGCTAGC TATGATCCGA ATTGAGAACC TCCCGGTGAA GGTCTTTGTG CTAAACAACC
AGCACCTGGG GATGGTGGTG CAGTGGGAGG ACAGGTTCTA TAAGGCCAAC AGAGCGCACA
CATACTTGGG AAACCCAGAG AATGAAAGTG AGATATATCC AGATTTCGTG ACGATCGCCA
AAGGGTTCAA CATTCCAGCG GTCCGTGTGA CAAAGAAGAA CGAAGTCCGC GCACGATAAA
GAAGATGCTC GAGACTCCAG GGCCGTACCT CTTGGATATA ATCGTCCCAC ACCAGGAGCA
TGTGTTGCCT ATGATCCCTA GTGGTGGGGC TTTCAAGGAT ATGATCCTGG ATGGTGATGG
CAGGACTGTG TACTGATCTA AAATCCAGCA AGCAACTGAT CTAAAATCCA GCAAGCACCG
CCTCCCTGCT AGTACAAGGG TGATATGTTT TTATCTGTGT GATGTTCTCC TGTGTTCTAT
CTTTTTTTGT AGGCCGTCAG CTATCTGTTA TGGTAATCCT ATGTAGCTTC CGACCTTGTA
ATTGTGTAGT CTGTTGTTTT CCTTCTGGCA TGTGTCATAA GAGATCATTT AAGTGCCTTT
TGCTACATAT AAATAAGATA ATAAGCACTG CTATGCGATG GTTCTGAAA
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FIG. 25

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CCTCGCGCCG CCTCCGAGAC AGCCGCCGCA ACCATGGCCA CCGCGGCCGC CGCGTCTACC
GGCCTCACTG GCCGCACTAC CGCTGCGCCC AAGGCAGGCG CCGCAGCCCT CCTGGCCACC
CGCCGCGCCC TCGCCGCGCC CATCAGGTGC TCAGCGGCGT CACCCGCCAT GCCGATGCGT
CCCCCGGCCA CCCCGCTCCG GCCGTGGGGC CCCACCGAGC CCCGCAAGGG TGCTGACATC
CTCGTCGAGT CCCTCGAGCG CTGCGGCGTC CGCGACGTCT TCGCCTACCC CGGCGGCGCG
TCCATGGAGA TCCACCAGGC ACTCACCCGC TCCCCCGTCA TCGCCAACCA CCTCTTCCGC
CACGAGCAAG GGGAGGCCTT TGCGCTCCGC GTACGCGCGC TCCTCGGCGC GCGTCGGCGT
CTGCATCGCC ACCTCCGGCC CCGGCGCCAC CAACCTAGTC TCCGCGCTCG CCGACGNCTG
CTCGATTCCG TCCCCATGGT CGCCATCACG GGACAGGTGC CGCGACGCAT GATTGGCACC
GACGCCTTCC AGGAGACGCC CATCGTCGAG GTCACCCGCT CCATCACCAA GCACAACTAC
CTGGTCCTCG ACGTCGACGA CATCCCCCGC GTCGTGCAGG AGGCTTTCTT CCTCGCCTCC
TCTGGTCGAC CAGGGCCGGT GCTTGTCGAC ATCCCCAAGG ACATCCAGCA GCAGATGGCG
GTGCCTGTCT GGGACAAGCC CATGAGTCTG CCTGGGTACA TTGCGCGCCT TCCCAAGCCC
CCTGCGACTG AGTTGCTTGA GCAGGTGCTG CGTCTTGTTG GTGAATCGCG GCGCCCTGTT
CTTTATGTGG GCGGTGGCTG CGCACGATCT GGTGAGGAGT TGCGACGCTT TGTGGAGCTG
ACTGGAATCC CGGTCACAAC TACTCTTATG GGCCTCGGCA ACTTCCCCAG CGACGACCCA
CTGTCTCTGC GCATGCTTGG TATGCATGGC ACGGTGTATG CAAATTATGC AGTGGATAAG
GCCGATCTGT TGCTTGCATT TGGTGTGCGG TTTGATGATC GCGTGACAGG GAAGATTGAG
GCTTTTGCAA GCAGGGCTAA GATTGTGCAC GTTGATATTG ATCCCGCTGA GATTGGCAAG
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FIG.25 cont.

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AACAAGCAGC CACATGTGTC CATCTGTGCA GATGTTAAGC TTGCTTTGCA GGGCATGAAT
GCTCTTCTTG AAGGAAGCAC ATCAAAGAAG AGCTTTGACT TTGGCTCATG GAACGATGAG
TTGGATCAGC AGAAGAGGGA ATTCCCCCTT GGGTATAAAA CATCTAATGA GGAGATCCAG
CCACAATATG CTATTCAGGT TCTTGATGAG CTGACGAAAG GCGAGGCCAT CATCGGCACA
GGTGTTGGGC AGCACCAGAT GTGGGCGGCA CAGTACTACA CTTACAAGCG GCCAAGGCAG
TGGTTGTCTT CAGCTGGTCT TGGGGCTATG GGATTTGGTT TGCCGGCTGC TGCTGGTGCT
TCTGTGGCAA ACCCAGGTGT CACTGTTGTT GACATCGATG GAGATGGTAG CTTTCTCATG
AACGTTCAGG AGCTAGCTAT GATCCGAATT GAGAACCTCC CGGTGAAGGT CTTTGTGCTA
AACAACCAGC ACCTGGGGAT GGTGGTGCAG TGGGAGGACA GGTTCTATAA GGCCAACAGA
GCGCACACAT ACTTGGGAAA CCCAGAGAAT GAAAGTGAGA TATATCCAGA TTTCGTGACG
ATCGCCAAAG GGTTCAACAT TCCAGCGGTC CGTGTGACAA AGAAGAACGA AGTCCGCGCA
GCATAAAGAA GATGCTCGAG ACTCCAGGGC CGTACCTCTT GGATATAATC GTCCCACACC
AGGAGCATGT GTTGCCTATG ATCCCTAATG GTGGGGCTTT CAAGGATATG ATCCTGGATG
GTGATGGCAG GACTGTGTAC TGATCTAAAA TCCAGCAAGC AACTGATCTA AAATCCAGCA
AGCACCGCCT CCCTGCTAGT ACAAGGGTGA TATGTTTTTA TCTGTGTGAT GTTCTCCTGT
GTTCTATCTT TTTTTGTAGG CCGTCAGCTA TCTGTTATGG TAATCCTATG TAGCTTCCGA
CCTTGTAATT GTGTAGTCTG TTGTTTTCCT TCTGGCATGT GTCATAAGAG ATCATTTAAG
TGCCTTTTGC TACATATAAA TAAGATAATA AGCACTGCTA TGCAGTGGTT CTGAAAAAAA
AAAAAA
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FIG. 26

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ACTGGCCGCA CTACCGCTGC GCCCAAGGCA GGCGCCGCAG CCCTCCTGGC CACCCGCCGC
GCCCTCGCCG CGCCCATCAG GTGCTCAGCG GCGTCACCCG CCATGCCGAT GCGTCCCCCG
GCCACCCCGC TCCGGCCGTG GGGCCCCACC GAGCCCCGCA AGGGTGCTGA CATCCTCGTC
GAGTCCCTCG AGCGCTGCGG CGTCCGCGAC GTCTTNGCCT ACCCCGGCGG CGCGTCCATG
GAGATCCACC AGGCACTCAC CCGCTCCCCC GTCATCGCCA ACCACCTCTT CCGCCACGAG
CAAGGGGAGG CCTTTCGCGC CTCCGCGTAC GCGCGCTCCT CGGCGCGCGT CGGCGTCTGC
ATCGCCACCT CCGGCCCCGG CGCCACCAAC CTAGTCTCCG CGCTCGCCGA CGCGCTGCTC
GATTCCGTCC CCATGGTCGC CATCACGGGA CAGGTGCCGC GACGCATGAT TGGCACCGAC
GCCTTCCAGG AGACGCCCAT CGTCGAGGTC ACCCGCTCCA TCACCAAGCA CAACTACCTG
GTCCTCGACG TCGACGACAT CCCCCGCGTC GTGCAGGAGG CTTTCTTCCT CGCCTCCTCT
GGTCGACCAG GGCCGGTGCT TGTCGACATC CCCAAGGACA TCCAGCAGCA GATGGCGGTG
CCTGTCTGGG ACAAGCCCAT GAGTCTGCCT GGGTACATTG CGCGCCTTCC CAAGCCCCCT
GCGACTGAGT TGCTTGAGCA GGTGCTGCGT CTTGTTGGTG AATCGCGGCG CCCTGTTCTT
TATGTGGGCG GTGGCTGCGC AGCATCTGGT GAGGAGTTGC GACGCTTTGT GGAGCTGACT
GGAATCCCGG TCACAACTAC TCTTATGGGC CTCGGCAACT TCCCCAGCGA CGACCCACTG
TCTCTGCGCA TGCTTGGTAT GCATGGCACG GTGTATGCAA ATTATGCAGT GGATAAGGCC
GATCTGTTGC TTGCATTTGG TGTGCGGTTT GATGATCGCG TGACAGGGAA GATTGAGGCT
TTTGCAAGCA GGGCTAAGAT TGTGCACGTT GATATTGATC CCGCTGAGAT TGGCAAGAAC
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FIG. 26 cont.

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AAGCAGCCAC ATGTGTCCAT CTGTGCAGAT GTTAAGCTTG CTTTGCAGGG CATGAATGCT
CTTCTTGAAG GAAGCACATC AAAGAAGAGC TTTGACTTTG GCTCATGGAA CGATGAGTTG
GATCAGCAGA AGAGGGAATT CCCCCTTGGG TATAAAACAT CTAATGAGGA GATCCAGCCA
CAATATGCTA TTCAGGTTCT TGATGAGCTG ACGAAAGGCG AGGCCATCAT CGGCACAGGT
GTTGGGCAGC ACCAGATGTG GGCGGCACAG TACTACACTT ACAAGCGGCC AAGGCAGTGG
TTGTCTTCAG CTGGTCTTGG GGCTATGGGA TTTGGTTTGC CGGCTGCTGC TGGTGCTTCT
GTGGCAAACC CAGGTGTCAC TGTTGTTGAC ATCGATGGAG ATGGTAGCTT TCTCATGAAC
GTTCAGGAGC TAGCTATGAT CCGAATTGAG AACCTCCCGG TGAAGGTCTT TGTGCTAAAC
AACCAGCACC TGGGGATGGT GGTGCAGTGG GAGGACAGGT TCTATAAGGC CAACAGAGCG
CACACATACT TGGGAAACCC AGAGAATGAA AGTGAGATAT ATCCAGATTT CGTGACGATC
GCCAAAGGGT TCAACATTCC AGCGGTCCGT GTGACAAAGA AGAACGAAGT CCGCGCNNNR
NTAAAGAAGA TGCTCGAGAC TCCAGGGCCG TACCTCTTGG ATATAATCGT CCCACACCAG
GAGCATGTGT TGCCTATGAT CCCTAGTGGT GGGGCTTTCA AGGATATGAT CCTGGATGGT
GATGGCAGGA CTGTGTACTG ATCTAAAATC CAGCAAGCAA CTGATCTAAA ATCCAGCAAG
CACCGCCTCC CTGCTAGTAC AAGGGTGATA TGTTTTTATC TGTGTGATGT TCTCCTGTGT
TCTATCTTTT TTTGTAGGCC GTCAGCTATC TGTTATGGTA ATCCTATGTA GCTTCCGACC
TTGTAATTGT GTAGTCTGTT GTTTTCCTTC TGGCATGTGT CATAAGAGAT CATTTAAGTG
CCTTTTGCTA CATATAAATA AGATAATAAG CACTGCTATG C
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I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/60; C12N9/88; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO,A,9 014 000 (ICI) 29 November 1990 see the whole document ---	2-6
A	NUCLEIC ACIDS RESEARCH. vol. 18, no. 8, 25 April 1990, ARLINGTON, VIRGINIA US page 2188; SATHASIVAN, K., ET AL.: 'Nucleotide sequence of a mutant acetolactate synthase gene from an imidazolinone-resistant Arabidopsis thaliana var. Columbia' see the whole document ---	1-6

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 FEBRUARY 1992	10. 03. 92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer MADDOX A.D.

2

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	PLANT PHYSIOLOGY. vol. 92, no. 4, April 1990, ROCKVILLE, MD, USA. pages 1081 - 1085; HAUGHN, G. W., ET AL.: 'A mutation causing imidazolinone resistance maps to the Csr1 locus of Arabidopsis thalina' see the whole document	2-6
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9101972
SA 53154

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